Journal of Bioenergetics and Biomembranes, Vol. 37, No. 6, December 2005 (© 2005)
DOI: 10.1007/s10863-005-9471-2

The Structural Basis for Coupling of Ca^{2+} Transport to ATP Hydrolysis by the Sarcoplasmic Reticulum Ca^{2+}-ATPase

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Recently, a series of structure determinations has nearly completed a structural description of the transport cycle of the sarcoplasmic reticulum Ca^{2+}-ATPase, especially those steps concerned with the phosphorylation by ATP and the dephosphorylation reaction. From these structures Ca^{2+}-ATPase emerges as a molecular machine, where globular cytosolic domains and transmembrane helices work in concert like a mechanical pump, as can be vividly demonstrated in animated versions of the pump cycle. The structures show that both ATP phosphorylation and dephosphorylation at Asp351 take place as nucleophilic SN2 reactions, which are associated with Ca^{2+} and H^+ occluded states, respectively. These transitory steps ensure efficient coupling between Ca^{2+} transport and ATP hydrolysis.

KEY WORDS: Ca^{2+}-ATPase; Ca^{2+} transport; sarcoplasmic reticulum; occlusion.

INTRODUCTION

P-type ATPase cation pumps are involved in the formation of electrochemical gradients for cations across biomembranes (Lutsenko and Kaplan, 1995; Møller *et al*., 1996). In addition to the large groups like Na^+, K^+-ATPase, Ca^{2+}-ATPases (SERCA, PMCA), and proton ATPases they comprise H^+,K^+-ATPase, responsible for gastric acid secretion, and ATPases involved in the transport of heavy metals like Cu as well as a number of other ATPases with so far ill-defined substrate specificities (Axelsen and Palmgren, 1998). P-type ATPases are distinguished from other active transporters like F-, V-, and ABC- ATPases by the formation of a covalent aspartylphosphorylated intermediate, of crucial importance for driving the active transport process by the conversion from an "energyrich" E1∼P state to the E2P state that is dephosphorylated by water. Among the many types and isoforms of P-ATPases the Ca^{2+}-ATPase present in skeletal muscle (SERCA 1a) assumes particular interest, because this ATPase for many years has been the subject of intense studies by biochemical and molecular biology techniques, and because of the recent success to produce crystals amenable to X-ray analysis at the atomic level: Out of a total of 183 entries in the Protein Data Bank for membrane proteins of known structure there is at present (as of December 2005) according to a protein database (White, 2005) seven entries in the Protein Data Bank representing the sarcoplasmic reticulum Ca^{2+}-ATPase in six different functional forms: 1SU4 (Ca_2E1), Toyoshima *et al*. (2000); 1IWO (E2(TG)), Toyoshima and Nomura (2002); 1T5S (Ca_2E1:AMPPCP) and 1T5T (Ca_2E1:ADP:AlF_4^-), Sørensen *et al*. (2004a); 1VFP (Ca_2E1:AMPPCP), Toyoshima and Mizutani (2004); 1WPG (E2(TG):MgF_4^{2-} Toyoshima *et al*., 2004; 1XP5 (E2(TG): AlF_4^-,) Olesen *et al*., 2004; all E2 forms stabilized by thapsigargin (TG)). The availability of these forms enables a detailed description of the structural changes accompanying a major part of the Ca^{2+} transport cycle. In the following we review and discuss the implications of these structures with particular emphasis on the processes leading to the phosphorylation and dephosphorylation of the Ca^{2+}-ATPase.

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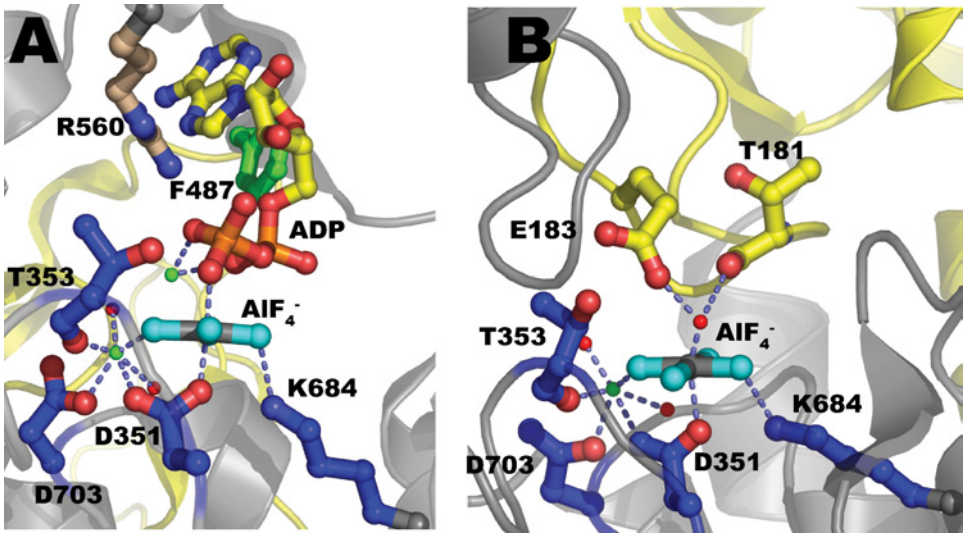

Fig. 1. Transitional states of sarcoplasmic reticulum Ca^{2+}-ATPase, corresponding to phosphorylation by ATP (A) and dephosphorylation (B). In Fig. 1(A) (taken from Sørensen *et al*., 2004a) phosphoryl transfer is mimicked by AlF_4^- complexed with Ca^{2+}-ATPase in the presence of Ca^{2+} and ADP (Ca_2E1:ADP: AlF_4^-), in Fig. 1(B) (taken from Olesen *et al*., 2004) dephosphorylation is mimicked by AlF_4^- complexed with Ca^{2+}-ATPase in the absence of bound Ca^{2+} (E2:AlF_4^-). The *green ball* seen in both Fig. 1(A) and (B) represents Mg^{2+} coordinated with Asp351, AlF_4^-, main chain Thr353, Asp703, and two water molecules (*red balls*); in Fig. 1(A) a second Mg^{2+} is also shown which is bound to the α,β-phosphate groups of ADP. It can be seen that in Fig. 1(B), with the Ca^{2+}-ATPase in the E2 conformation, a water molecule, coordinating AlF_4^- with Glu183 and Thr181 functions as the nucleophilic substituent which replaces ADP and forms the coordinated complex with AlF_4^- in the E1 conformation (Fig. 1(A)).

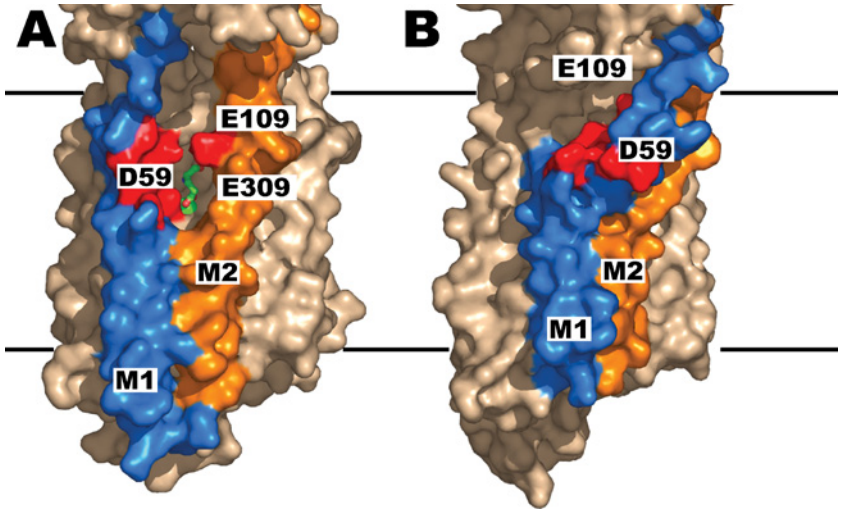

Fig. 2. A putative N-terminal entrance for Ca^{2+} (Fig. 2(A)) that is blocked in the occluded state (Fig. 2(B)): Fig. 2(A) shows the transmembranous Ca^{2+}-ATPase domain in the Ca_2E1 conformation where both M1 (*blue*) and M2 (*orange*) follow a straight course through the membrane; while Fig. 2(B) shows Ca^{2+}-ATPase as the Ca_2E1:ADP: AlF_4^- complex where M1 is kinked and rotated so that it blocks a putative entrance to Glu309 (*green sticks*) and Ca^{2+} bound at the Site 2 (*green ball*) that are visible in Fig. 2(A). *Red color* designates residues present at the cytosolic border, including Asp59 in M1 and Glu109 in M2.

PHOSPHORYLATION AND OCCLUSION OF Ca^{2+}

The Ca^{2+} transport cycle starts with the Ca^{2+} dependent phosphorylation by ATP, leading to the formation of the $Ca_2E1{\sim}P$ high-energy intermediate, phosphorylated at Asp351, and accompanied by the occlusion of the two intramembraneously bound Ca^{2+} ions. During the reaction of $Ca_2E1{\sim}P$ with ATP, the ATPase with its 10 transmembrane helices and cytosolic P(phosphorylation)-, N (nucleotide binding)-, and A(actuator)- domains is converted from an open to a compact structure (Sørensen *et al*., 2004a; Toyoshima *et al*., 2004). This is the result of crosslinking of the N- and P-domains by the bound ATP whereby the γ-phosphate is brought in close contact with the phosphorylatable Asp351 residue. As shown in Fig. 1(A), ADP in the ADP: AlF_4^- complex is bound in a characteristically bent conformation by residues derived from the N-domain, while AlF_4^-, as a representative of the γ-phosphate in ATP, forms a nearly linear complex with the β-phosphate of ADP and the carboxyl group of Asp351 with distances between the involved oxygen atoms being around 2 Å. These features are characteristic of an SN2 nuclear associative reaction mechanism that with phosphate replacing AlF_4^- would lead to transfer of the covalent bond from ATP to the carboxyl group of Asp351. Of further importance for the reaction to proceed is the positive charge at the phosphorylation site delivered by Lys-684 and by Mg^{2+} (or Ca^{2+} as a substitute of Mg^{2+} under the conditions of high Ca^{2+} concentrations (10 mM) used for crystallization (Jensen, Picard, Sørensen, Møller, and Nissen, unpublished data)). In addition to Asp351 and AlF_4^-, the Mg^{2+} at the phosphorylation site is coordinated with other nearby amino acid residues (main chain Thr353 and Asp703). Finally, the structure is characterized by the presence of a second bound Mg^{2+} ion which coordinates with the α,β-phosphates and Arg-560 in the N-domain. We consider that this second bound Mg^{2+} reflects the formation of MgADP (rather than ADP) as the leaving substrate from the transition state complex.

An almost identical structure was obtained after the binding of AMPPCP as a nonhydrolyzable analogue of ATP (Sørensen *et al*., 2004a). The nucleotide is present inside the binding cavity formed between the N- and P-domain in the same conformation as in the ADP: AlF_4^- complex with only slightly longer bond distance of the γ-phosphate to Asp351 and with Mg^{2+} (or Ca^{2+}) that is coordinated in the same way at the phosphorylation site as in the ADP: AlF_4^- complex (but the complex lacks the coordination of the second Mg^{2+} with the N-domain). In both structures nucleotide binding, leading to N/P domain crosslinking, evidently occurs without perceptibly affecting the intramembranal Ca^{2+} binding environment as present in the Ca_2E1 open structure. However, the N/P domain interaction sets the stage for a significant change in the disposition of the A-domain which is caused by the formation of a notch between the 576–581 loop of the N-domain and the 720–725 loop of the P-domain that is fit for interaction with the 155–160 residues of the A-domain (Sørensen *et al*., 2004a). To realize this interaction the A-domain has to perform a 45° upward rotation which in turn exerts a direct pull on the M1 and M2 transmembrane segments. Unlike the M4–6 and M8 segments, M1 and M2 are not directly engaged in the intramembraneous liganding of Ca^{2+}, and as a consequence of the pull M2 is lifted up 8–9 Åfrom the membrane, resulting in a transfer of its C-terminal residues into the cytosolar space and withdrawal of luminal residues between M1 and M2 into the membrane. In M1 the strongly amphipatic (^{54}I E Q F E D L)- N-terminal part is extracted from the hydrophobic membrane phase, but rather than being lifted up it forms a kink to a assume a thermodynamically more relaxed state lying flat with the N-terminal half on the nonpolar membrane interface (Fig. 2(B)). As a result of these movements a putative N-terminal entrance in the Ca_2E1 state to the intramembranous Ca^{2+} binding sites between M1 and M2 (Fig. 2(A)) is closed.

During the Ca^{2+} transport cycle an occluded state is a prerequisite to obtain an efficient 2:1 coupling between Ca^{2+} transport and ATP hydrolysis. In the physiological function of the Ca^{2+} pump occlusion is an ephemeral state, the existence of which mainly has been inferred from evidence for release of bound Ca^{2+} toward the luminal side after phosphorylation by ATP (Dupont, 1980; Takisawa and Makinose, 1983). The biochemical experiments that we performed on membranous Ca^{2+}-ATPase alongside with the structural analysis of the ADP: AlF_4^- ATPase crystalline complex confirmed previous data (Troullier *et al*., 1992) that Ca^{2+} in this complex is bound in an occluded state, in agreement with the structural evidence for occlusion described above. On the other hand, also in agreement with earlier kinetic observations (Orlowski and Champeil, 1991), we confirmed that the AMPPCP bound ATPase, despite the very similar crystal structure is unable to occlude Ca^{2+}. Furthermore, as compared to the ADP:AlF_4^- complex, the AMPPCP bound ATPase retained the ability of unliganded ATPase to react with −SH reagents and antibodies, and also to become degraded by proteolytic enzymes as reported by Danko *et al*. (2001). We therefore consider that, as a consequence of the reversibility of the binding process, the AMPPCP bound ATPase in the native membraneous state is present as a dynamically fluctuating structure, which only in the crys-

tal form becomes trapped in an occluded state. A kinetic reinvestigation by Picard *et al.* (2005) suggests that the high concentrations of Ca^{2+} used in the crystallization studies (5–10 mM) could contribute to stabilization of this state.

The important conclusion to accrue from all of these findings is that Ca^{2+} occlusion during the normal transport cycle is confined to the short period of the ATP phosphorylation transition state, but that this state can be stabilized by the use of structural analogues such as ADP:AlF_4^- or CrATP (Coan *et al.*, 1994; Vilsen and Andersen, 1992). The background for occlusion in these states probably is that the formation of the ADP:AlF_4^-:ATPase or CrATP:ATPase complexes poses very stringent requirements on the conformation of the ATPase, not only at the phosphorylation site, but also in the remaining parts of the ATPase molecule, including the membranous domain. Therefore the transition state leads to a "frozen" conformation and occlusion, which in the physiological mode of the pump ends with the completion of the ATP phosphorylation process and the removal of MgADP to produce the following state where the ATPase is ready for translocation of Ca^{2+}.

E2P DEPHOSPHORYLATION

In connection with Ca^{2+} translocation the Ca^{2+}-ATPase is converted to the E2P state with opening of the luminal gate, which leads to the release of Ca^{2+} on the other side of the membrane by an alternating access mechanism. It has been found that this transition is accompanied by a pronounced decrease in the affinity of the membranous binding sites for Ca^{2+} (Andersen *et al.*, 1985) that accounts for the ability of the ATPase to actively transport Ca^{2+} against a concentration gradient (Tanford, 1983). We do not as yet have exact structural information on the details of this important transition which is powered by conformational changes transmitted from the cytosolic domains to the membrane, where the A-domain in conjunction with the P-domain seems to play the essential roles (Møller *et al.*, 2002; Wang *et al.*, 2005). On the other hand, we have obtained structures pertaining to the following E2P dephosphorylation reaction where, in the absence of Ca^{2+}, AlF_4^- binds to the ATPase as a transitional state analog of E2P (Danko *et al.*, 2004), the structure of which has been solved after stabilization of the Ca^{2+}-ATPase by thapsigargin.(Olesen *et al.*, 2004). An essential feature of the E2: AlF_4^- structure is that the A-domain has moved into close contact with the P-domain, leading to partial removal of the N-domain. This movement is achieved by a 110° rotation around an axis normal to the membrane which by a further translational movement brings the conserved TGES motif present in this domain in contact with the phosphorylation site. Here, the Glu183 and Thr181 residues of this motif via a bridging water molecule interact with AlF_4^- as shown in Fig. 1(B). The bridging water molecule is located for an inline attack so that it can catalyze the dephosphorylation reaction by an associative SN2 mechanism where Glu183 by abstraction of a proton from the bound water molecule is involved as a general base catalyst. This role of Glu183 has recently been confirmed by site-directed mutagenesis (Clausen *et al.*, 2004).

Another intermediary structure has been reported by Toyoshima *et al.* (2004) where the thapsigargin-stabilized ATPase was reacted with MgF_4^{2-} to produce a complex mimicking the E2P dephosphorylation product state. From a detailed comparison with the Ca_2E1:ADP:AlF_4^- structure, M3 and M4 were concluded to be deeper inserted in the membrane, causing displacements of the M1 and M2 transmembrane segments, but with retention of the characteristic kink of the N-terminal part of the M1 helix. In the cytosolic regions the same movements of the N-, P-, and A-domain were observed as in our study. MgF_4^{2-} was also found to interact with Asp351 and Glu183 of the TGES motif, but the interaction seemed to include Gly182, rather than Thr181 as in our structure. A characteristic feature of both structures is that, as a consequence of the movement of the N-domain and rotation of the A-domain, the TGES motif has replaced bound nucleotide at the phosphorylation site in the Ca_2E1:ADP:AlF_4^- complex. In addition to providing a plausible mechanism for the dephosphorylation mechanism the presence of the TGES motif at the phosphorylation site also gives a direct structural explanation of the ADP insensitivity of the E2P state.

A consequence of the rotation of the A-domain is that the A-M3 arm, i.e. the polypeptide chain linking the A domain with M3, moves from a peripheral and loosely attached position in the Ca_2E1P conformation toward the main body of the Ca^{2+}-ATPase in the E2P state. Here, together with the corresponding A-M2 arm it forms a tight five-helical cluster with the C-terminal helices of the P-domain (P4–P6). The involvement of this helical association in dephosphorylation is suggested by a severe and specific inhibition of the dephosphorylation reaction after proteolytic cleavage of the A-M2 arm (Lenoir *et al.*, 2004) while after cleavage of the A-M3 arm it is primarily E2P formation that is curtailed (Møller *et al.*, 2002). Furthermore, we have detected a specific binding site for K^+ (Rb^+) at Glu732 and Lys712 that seems to be of importance for the close association of the helical cluster and the well-known allosteric stimulation by K^+

(Shigekawa and Pearl, 1976) of the E2P dephosphorylation rate (Sørensen *et al*., 2004b). Probably an important function of the cluster is to consolidate the interaction of the TGES motif with the phosphorylation site and thereby assist in the dephosphorylation process. After hydrolysis of phosphate the TGES motif is retracted from the phosphorylation site and the helical cluster assumes a more relaxed state (Olesen *et al*., 2004).

Ca^{2+}/H^+ EXCHANGE

It is generally acknowledged that the sarcoplasmic reticulum Ca^{2+}-ATPase normally functions as an electrogenic Ca^{2+}/H^+ exchanger (Levy *et al*., 1990) where the protons partially compensate for the positive electrostatic charge (Cornelius and Møller, 1991; Yü *et al*., 1993) associated with the intravesicular uptake of Ca^{2+}: depending on medium conditions 2–3 H^+ appear to be taken up in exchange for the 2 Ca^{2+} delivered to the luminal space (Yü *et al*., 1994). Our E2: AlF_4^- structure is in agreement with the view that in the E2P transition state protons are present inside the membrane in an occluded state (Forge *et al*., 1993; Wakabayashi and Shigekawa, 1990). The occluded protons cannot be seen directly, but their presence in the E2:AlF_4^- structure can be deduced from the fact that all the carboxylate liganding groups (Glu771, Asp800, Glu908, and Glu309) are located inside the nonpolar membrane environment in a state where no Ca^{2+} or other metal ions are present to neutralize their charge. In accordance with the concept of the transition state representing an occluded state the binding cavity is separated from the lumen by a dense, 15 Å thick barrier mainly consisting of the hydrophobic and some basic residues formed by the luminal half of the Ca^{2+}-ATPase transmembrane helices. The barrier toward the lumen clearly distinguishes the structure from the E2P ground state, where these binding sites are expected to be in contact with the intraluminal medium for Ca^{2+}/H^+ exchange to take place. Among the negatively charged intramembranous residues the centrally placed Asp800 and Glu771 residues are likely candidates for an obligatory charge neutralization by protons from the luminal space: Compared to these residues Glu309 is in a more exposed position toward the cytosolic space and could be present in at least a partially ionized state, while Glu908 according to molecular dynamic simulation calculations performed by Sugita *et al*. (2005), is likely to be protonated even in the Ca_2E1 state. In conclusion, the structure is consistent with the occlusion of 2–3 luminal protons per 2 Ca^{2+} transported. As in the case of Ca^{2+} occlusion, the biochemical evidence on membranous Ca^{2+}-ATPase complexed with AlF_4^- alone suggests that in the E2P transition state the ATPase is in a "frozen" conformation. This view is supported by a sluggish reactivation of the complex, even by high Ca^{2+} concentrations, a resistance to proteolytic digestion, and a low −SH and antibody reactivity. As in the case of the Ca_2E1:ADP:AlF_4^- complex, the stabilization of the protein structure can be rationalized on the basis of the stringent requirements needed to maintain the TGES motif in the A-domain in the correct position for an inline attack on the phosphorylated Asp351 residue.

COMPLETION OF THE CYCLE

After dephosphorylation the compact conformation of the ATPase is retained, as indicated by the E2 (1 IWO) structure, stabilized by thapsigargin, in the absence of Ca^{2+} and nucleotide (Toyoshima and Nomura, 2002). Maintenance of the compact state in the absence of "cross-linking" nucleotide signifies the persistence of interactions between the A-domain and P-domain, despite that the TGES motif is retracted from its close interaction with the phosphorylation site. Probably this interaction is aided by retention of the aforementioned cluster of helices formed between the C-terminal helices in the P-domain with the A-M2 and A-M3 arms. In addition the N-domain is slightly less tilted by movement toward the P-domain, but otherwise the structure, including the rotation of the A-domain toward the P-domain, is remarkably similar to that of both the E2:AlF_4^- and E2:MgF_4^- forms. The M1 helix is still kinked as in the E1:ADP:AlF_4^- form, but due to the rotation of the A-domain, an N-terminal opening to the membrane interior is formed, lined by polar residues above the kink and the M2- and M3 extensions of the M2/M3 transmembrane helices (Toyoshima and Nomura, 2002). This entrance leads directly to Glu309, which in addition to its role as a Ca^{2+} binding residue at site 2 also is considered to play an important role to function as a cytosolar gate, to allow the release of protons and the uptake of Ca^{2+}.

We have found it possible to prepare crystals of the above described compact E2 conformation with bound AMPPCP (Jensen, Sørensen, Olesen, Møller, and Nissen, unpublished observations). As might be expected the A-domain blocks for interaction of the bound nucleotide at the phosphorylation site. Instead the Glu439 residue of the N-domain via a bridging Mg^{2+} interacts with the α,β-phosphate groups of ATP. This mode of binding is consistent with iron-cleavage data showing that binding of FeATP to ATPase as a substitute MgATP leads to peroxidative cleavage at Glu439 (Hua *et al*., 2002; Montigny *et al*., 2004). The γ-phosphate is bound on the outskirts of the P-domain near the conserved

^{625}TGD motif. In the membranous region the position of Glu309 is rotated toward the interior of the membrane so that it does not obstruct the N-terminal entrance as has been reported when nucleotide is absent (Toyoshima and Nomura, 2002). Since the ATPase normally operates in the presence of millimolar concentrations of ATP, it is likely that Ca^{2+} interacts with E2 *after* binding of nucleotide in such a way that the compact conformation will be fully or partially maintained all the way during the conversion of the enzyme to the E1 state with bound Ca^{2+} and nucleotide. This more direct route can be expected to accelerate the rate of phosphorylation, compared to that which would occur via the open Ca_2E1 (1SU4) form by the following set of reactions: (1) E2 $\rightarrow$ E1; (2) E1 + 2 Ca $\rightarrow$ Ca_2E1 (open form); (3) Ca_2E1 + ATP $\rightarrow$ Ca_2E1$\sim$P + ADP (compact form). In other words prebinding of crosslinking nucleotide may obviate some of the conformational changes otherwise required for the transition from the E2 state to the ATP phosphorylated Ca_2E1$\sim$P state and thereby account for some of the modulatory effects of ATP on the Ca^{2+} transport cycle.

ACKNOWLEDGMENTS

This work has been supported by grants from the Danish Medical Research Council, the Lundbeck Foundation, the Aarhus University Resesarch Foundation, and the Novo Nordic and P. C. Petersen Foundation. We wish to thank our colleagues, and especially Philippe Champeil (CEA, Saclay), for many discussions of the functional significance of our data.

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