The Cytoplasmic Loop Located between Transmembrane Segments 6 and 7 Controls Activation by Ca$^{2+}$ of Sarcoplasmic Reticulum Ca$^{2+}$-ATPase*

(Received for publication, March 18, 1998, and in revised form, May 18, 1998)

Thierry Menguy‡, Fabienne Corre‡§, Laurence Bouneau‡∥, Stéphane Deschamps‡∥, Jesper Vuust Moller‡∥∥, Philippe Champeil‡, Marc le Maire‡, and Pierre Falson‡ ‡‡

From the ¶€Section de Biophysique des Protéines et des Membranes, DBCM, Commissariat à l'Energie Atomique and CNRS URA 2096, CE Saclay, 91191 Gif sur Yvette Cedex, France and the **Danish Biomembrane Research Centre, Department of Biophysics, University of Aarhus, DK-8000 Aarhus C, Denmark

During active cation transport, sarcoplasmic reticulum Ca$^{2+}$-ATPase, like other P-type ATPases, undergoes major conformational changes, some of which are dependent on Ca$^{2+}$ binding to high affinity transport sites. We here report that, in addition to previously described residues of the transmembrane region (Clarke, D. M., Luo, T. W., Inesi, G., and MacLennan, D. H. (1989) Nature 339, 476–478), the region located in the cytosolic L6–7 loop connecting transmembrane segments M6 and M7 has a definite influence on the sensitivity of the Ca$^{2+}$-ATPase to Ca$^{2+}$, i.e. on the affinity of the ATPase for Ca$^{2+}$. Cluster mutation of aspartic residues in this loop results in a strong reduction of the affinity for Ca$^{2+}$, as shown by the Ca$^{2+}$ dependence of ATPase phosphorylation from either ATP or Pi. The reduction in Ca$^{2+}$ affinity for phosphorylation from Pi is observed both at acidic and neutral pH, suggesting that these mutations interfere with binding of the first Ca$^{2+}$, as proposed for some of the intramembranous residues essential for Ca$^{2+}$ binding (Andersen, J. P. (1995) Biosci. Rep. 15, 243–261). Treatment of the mutated Ca$^{2+}$-ATPase with protease K, in the absence or presence of various Ca$^{2+}$ concentrations, leads to Ca$^{2+}$-dependent changes in the proteolytic degradation pattern similar to those in the wild type but observed only at higher Ca$^{2+}$ concentrations. This implies that these effects are not due to changes in the conformational state of Ca$^{2+}$-free ATPase but that changes affecting the proteolytic digestion pattern require higher Ca$^{2+}$ concentrations. We conclude that aspartic residues in the L6–7 loop might interact with Ca$^{2+}$ during the initial steps of Ca$^{2+}$ binding.

Sarcoplasmic reticulum (SR) Ca$^{2+}$-ATPase belongs to the family of P-type cation-transporting ATPases that transport cations by an active mechanism involving the formation of a phosphorylated intermediate (1–4). Members of this large family have been classified as type I or type II ATPases, corresponding to proteins transporting, respectively, heavy metals or cations such as H$^+$, Na$^+$, K$^+$, or Ca$^{2+}$ (5). Ca$^{2+}$-ATPase in mature skeletal muscle contains 994 residues, as deduced from cloning and sequencing of the SERCA1a gene by MacLennan and co-workers (6). According to a topological model based on both sequence-derived predictions and experimental protein-chemical data, 70% of the polypeptide consists of two cytosolic domains connected to the membrane-embedded part by a stalk of putative helices. The membranous part comprises about 20% of the residues, arranged in 10 putative transmembrane spans, M1–10, with 10% additional residues that could form a small luminal domain. Structural three-dimensional data, obtained to date only at low resolution, have confirmed this description of the general organization of the protein (7), but the detailed structure of the Ca$^{2+}$-ATPase remains an open question (8, 9).

During Ca$^{2+}$ transport, Ca$^{2+}$-ATPase probably undergoes several conformational changes, although only two main states were initially considered (1), which denote forms with a high and low affinity for Ca$^{2+}$, respectively (see also Refs. 9 and 10). Binding of cytosolic Ca$^{2+}$ at the Ca$^{2+}$-activating and transport sites of the ATPase was measured directly under equilibrium conditions and found to occur with a stoichiometry of 2:1 and a positive cooperativity, which was interpreted on the basis of a sequential mechanism (11). It has been proposed to occur in a multistep process accompanied by conformational changes. Evidence has been presented that binding of only one Ca$^{2+}$ is sufficient to prevent phosphorylation from Pi, while the binding of the second Ca$^{2+}$ is required to allow subsequent phosphorylation from ATP (12). Mainly based on the kinetics of Ca$^{2+}$ dissociation, Ca$^{2+}$-binding sites have been suggested to be ar-

* This work was supported by Commissariat à l’Energie Atomique (CEA) and CNRS, by “Ministère de la Recherche et de la Technologie” fellowships (to T. M.), and by “Association Franc¸aise contre les Myopathies” (CEA) and CNRS, by “Ministe`re de la Recherche et de la Technologie” (Ministry of Research and Technology). This article must therefore be hereby marked “in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence and reprint request should be addressed: falson@dsvidf.cea.fr.

1 The abbreviations used are: SR, sarcoplasmic reticulum; SERCA, sarcoplasmic reticulum Ca$^{2+}$-ATPase; p85, C-terminal protolytic fragment of SR Ca$^{2+}$-ATPase starting at residue Lys-120; p83C, C-terminal protolytic fragment of SR Ca$^{2+}$-ATPase starting at residue Asp-818; C$_E$E$_N$, octaethylene glycol monododecyl ether; Tes, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethylamino]ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; Ab, Antibody; WT, wild type; ADA, aspartate mutant D813A/D818A; AAA aspartate mutant D813A/D815A/D818A; PDDF, polyvinylidine difluoride; MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpholineethanesulfonic acid; E$_c$, conformational state of Ca$^{2+}$-ATPase of high affinity for calcium; E$_o$, conformational state of Ca$^{2+}$-ATPase of low affinity for calcium.
ranged in a channel-like structure including two sites (13–15). After phosphorylation with ATP, the Ca$^{2+}$ ions can initially no longer dissociate from the highly energy phosphoenzyme formed and are found in what has been described as an “occluded” state (16, 17). The Ca$^{2+}$ ions are subsequently released toward the SR lumen because of reorganization of both sites and loss of their affinity for Ca$^{2+}$ (18–20). It has been shown that after this stage, protons (or hydronium ions) may combine with and be countertransported by the enzyme (21, 22). Chemical and/or genetic modification of the polypeptide chain has allowed us to examine the functional role of different domains (for reviews, see Refs. 5, 10, 23, and 24). Residues critical for Ca$^{2+}$ binding and transport were not found in the stalk sector, despite the fact that this region has a large content of acid residues (25). Instead, such residues are clustered in the transmembrane domain, in M4 (Glu-309), M5 (Glu-771), M6 (Asn-796, Thr-799, and Asp-800) and M8 (Glu-908) (26). Further experiments showed that the five residues in M4, M5, and M6 were also critical for occlusion in the presence of Cr-ATP (27), but not Glu-908, which was therefore considered to be involved in the initial recognition of the Ca$^{2+}$ ions but not in its final intramembranous binding (10, 28).

In addition to the molecular biology approach, limited proteolysis of the SR Ca$^{2+}$-ATPase has also been developed for identification of regions critical for Ca$^{2+}$ activation of the ATPase (29–31). Experiments showed that the rate of electrophoretic migration of a small C-terminal ATPase peptide, p20C, was sensitive to Ca$^{2+}$, while a slightly shorter peptide, p19C, was not. We found that p20C starts at Gly-808, at the beginning of the loop connecting putative transmembrane spans 6 and 7, while p19C starts at Asp-818, in the middle of loop. This led us to suggest that the L6–7 loop might interact with Ca$^{2+}$ (32). The N-terminal part of the loop contains three aspartic residues (Asp-813, -815, and -818), which were mutated in a cluster, D813A/D815A and D813A/D815A/D818A. After expression in yeast, we found that these mutants had a lower Ca$^{2+}$-ATPase activity (32). It is noteworthy that in gastric H+–K+–ATPase, residues Glu-837 and Asp-839, which correspond to Asp-813 and Asp-815 in Ca$^{2+}$-ATPase, were found to render the ATPase unphosphorylatable by ATP when mutated to glutamine and asparagine residues, respectively (33).

In the present work, we have carried out more detailed experiments to investigate the role of the L6–7 loop in Ca$^{2+}$-ATPase. The experiments were designed to characterize the functional consequences of mutations of aspartic residues, D813A/D815A and D813A/D815A/D818A, as well as those of mutations of the proline residues in the same loop, Pro-811, -812, -820, and -821. We found that cluster mutations of aspartic residues led to a clear reduction in the apparent affinity with which Ca$^{2+}$ controlled ATPase phosphorylation or dephosphorylation. Although Asp to Ala mutations are often thought of as conservative, susceptibility to proteolytic digestion suggested that our mutation of the L6–7 loop did not cause any major conformational change. Thus, the present findings are consistent with an interaction of the aspartic residues of the L6–7 loop with the first Ca$^{2+}$ ion that binds to Ca$^{2+}$-ATPase during the transport process and suggest that the L6–7 loop contributes to the control of the activation of Ca$^{2+}$-ATPase by Ca$^{2+}$ during the initial steps of Ca$^{2+}$ binding. A model is proposed describing one possible mechanism by which such a control might occur.

MATERIALS AND METHODS

Mutation and Expression of Ca$^{2+}$-ATPase in Yeast—The single mutants E309Q and E771Q and the cluster mutants D813A/D818A (referred to later as AAA) and D813A/D815A/D818A (referred to later as AAA) were obtained as in Ref. 32. Using Ca$^{2+}$-ATPase SERCA1a cDNA (34), single mutants P812A and P821A and cluster mutants P811A/ P812A and P820A/P821A were obtained by site-directed mutagenesis performed with the pAlter kit (Promega). The presence of the desired mutations and the absence of unexpected ones resulting from the polynucleotide chain reaction were verified by DNA sequencing. Wild type and mutant DNA were inserted into a yeast expression vector using the polynucleotide chain reaction (31) and a GS-700 imaging densitometer with Molecular Analyst software (Bio-Rad); for reference, we used native SR membranes in which 75% of the protein content is assumed to be Ca$^{2+}$-ATPase. Typically, light membranes expressed wild type or mutant Ca$^{2+}$-ATPase at about 0.75 mg of Ca$^{2+}$-ATPase/100 mg of membrane proteins.

ATPase Assay—ATP hydrolysis was assayed spectrophotometrically at 30 °C as in Ref. 32, generally with 50 μg of yeast light membranes/ml in the assay and with a final Ca$^{2+}$ concentration of 0.1 mM. The reaction was started by the addition of 1 mM Na$_2$ATP. Thapsigargin (1 μg/ml; see Ref. 41) was added after 100 s, and the rate of hydrolysis was followed for an additional 100 s. The Ca$^{2+}$-ATPase activity was calculated as the difference between the slopes obtained in the presence and in the absence of thapsigargin and was corrected for the (very small) background of thapsigargin-dependent activity obtained with light membranes of control yeasts.

Ca$^{2+}$ Dependence of Ca$^{2+}$-ATPase Phosphorylation from [γ$^{32}$P]ATP, as Measured by a Filtration Method—Ca$^{2+}$-ATPase phosphorylation from [γ$^{32}$P]ATP was carried out in an ice-cold buffer containing 20 mM MOPS-Tris, pH 7, 100 mM KC1, 5 mM MgCl$_2$, and various free Ca$^{2+}$ concentrations, as indicated in Figs. 3 and 4. Final Ca$^{2+}$ (obtained with mixing various amounts of EGTA) were calculated using Maxchelator software, taking into account the endogenous Ca$^{2+}$. Five mM Na$_2$S$_2$O$_3$, 0.05 μM bafloimycin A, and 0.1 mM ammonium molybdate were also included in the assay medium to inhibit other ATPases. Fifty μg of light yeast membrane proteins were added to a final volume of 50 μl. The reaction was started by adding 2 μl [γ$^{32}$P]ATP (1 Ci/mmol) and quenched after 15 s by adding 2 ml of cold quenching solution containing 150 mM perchloric acid and 15 mM Na$_2$PO$_4$. Acid-denatured proteins were retained on a glass fiber filter (Gelman, AE type) and washed five times with 4 ml of quenching solution (36). The radioactivity in the filter was then counted in 4 ml of scintillation liquid (Packard, Filter-Count). A filter containing 100 pmol of [γ$^{32}$P]ATP was used as a standard, to convert measured cpm to pmol.

Ca$^{2+}$ Dependence of Ca$^{2+}$-ATPase Phosphorylation from [γ$^{32}$P]ATP, as Measured After Electrophoretic Separation—Ca$^{2+}$-ATPase phosphorylation was carried out as in the case of the filtration experiments except that proteins were diluted 10 times more (i.e. into a final volume of 500 μl with the same buffer), and the various free Ca$^{2+}$ concentrations were established more reliably, by mixing Ca$^{2+}$ with EGTA and Mg$^{2+}$EDTA. In this case, the reaction was stopped by adding 1 ml of a cold solution containing 9% trichloroacetic acid and 27 mM Na$_2$PO$_4$. Acid-denatured proteins were treated according to Ref. 38 and sedimented by centrifugation for 15 min at 18,000 rpm (25,000 × g$_{rot}$) at 4 °C. The pellet was neutralized by the addition of 1 μl of 1 M Tris-base and then suspended in 50 μl of 150 mM Tris-Cl, pH 6.8, 10 mM EDTA, 2% SDS, 16% glycerol (w/v), 0.04% bromphenol blue (v/v), and 0.84 μl β-mercaptoethanol. After 10 min at room temperature, proteins corresponding to 150 ng of Ca$^{2+}$-ATPase were loaded on 7% polyacrylamide gels and run for 90 min at 120 V in 170 mM MOPS-Tris, pH 6.0, 0.1% SDS. After electrophoresis, the gels were fixed in 45% methanol and 10% acetic acid for 25 min and then dried overnight between two sheets of cellophane paper. Radioactivity was revealed with a Phosphorimage (Molecular Dynamics, Inc.) and a Biomax film (Amersham Pharmacia Biotech) and quantified by comparison with known amounts of [γ$^{32}$P]ATP.

Ca$^{2+}$ Dependence of Ca$^{2+}$-ATPase Phosphorylation from [γ$^{32}$P]P—Phosphorylation from P$^32$ was assayed both at pH 6.0 and at pH 7.0. The reaction was carried out at 20 °C in a total volume of 500 μl of 100 mM MES-Tris, pH 6.0, or 100 mM MOPS-Tris, pH 7.0, 5 mM MgCl$_2$, 20% dithiothreitol, 5 mM Na$_2$S$_2$O$_3$, 0.05 μM bafloimycin A, 0.1 mM ammonium molybdate, also containing various mixtures of Ca$^{2+}$ and EGTA or MgEDTA to obtain the free Ca$^{2+}$ concentrations indicated in Figs. 5 and 6. The reaction was started by adding 50 μg of light membranes (corresponding to 375 ng of Ca$^{2+}$-ATPase) and 0.1 mM [γ$^{32}$P]ATP, and stopped after 15 s by the addition of 2 ml of cold quenching medium (9% trichloroacetic acid, 27 mM Na$_2$PO$_4$). Quantification of...
to 150 ng of Ca²⁺ buffer (39) and heated for 1 min at 100 °C, and aliquots corresponding to two couples of proline residues indicated by squares. These seven residues have been mutated in this work.

Results
Choice of Mutations in the L6–7 Loop—The primary structure of the L6–7 loop is shown in Fig. 1. The N-terminal region of the loop, which is highly conserved among SERCA ATPases, presents two remarkable features; it contains three aspartic residues, Asp-813, Asp-815, and Asp-818 (note that conservative replacements with Glu or Asn residues are found for Asp-813, Asp-815, and Asp-818) previously suggested to be of interest for Ca²⁺ binding (32) are indicated by triangles. They are surrounded by two couples of proline residues indicated by squares. These seven residues were mutated in this work.

Asp substitutions with the aim of suppressing all potential calcium-liganding oxygen atoms in the side chain. A few single mutations (D813A and D815A) were also tested (see "Ca²⁺-ATPase Activities of Mutants"). All mutants were prepared by site-directed mutagenesis and expressed in yeast as described under "Materials and Methods." The results presented below were obtained using a yeast membrane protein of wild type or native (SR) ATPase and also with those for two previously described mutants, E309Q and AAA, compared with those for the WT or native (SR) ATPase and also with those for two previously described mutants, E309Q and AAA (31) while the C-terminal end comprises Gly-831 (S. Soulé, S., DeForesa, B., Møller, J. V., Bloomberg, G. B., Groves, J. D., LeMaire, M., manuscript in preparation). The aspartic residues (Asp-813, Asp-815, and Asp-818) previously suggested to be of interest for Ca²⁺ binding (32) are indicated by triangles. They are surrounded by two couples of proline residues indicated by squares. These seven residues have been mutated in this work.

An initial Ca²⁺-binding site, located at the membrane-cytosol interface and structured by the proline residues. Therefore, these residues were mutated to alanine residues to give the following mutants: D813A/D818A (referred to as ADA), D813A/D818A (referred to as AAA), P811A/P812A, P812A, P820A/P821A, and P821A. Cluster replacement of the negatively charged Asp residues by the small neutral Ala residues was performed in preference to the a priori more conservative Asp → Asn or Asp → Glu substitutions with the aim of suppressing all potential calcium-liganding oxygen atoms in the side chain. A few single mutations (D813A and D815A) were also tested (see "Ca²⁺-ATPase Activities of Mutants").
several ATPases inhibitors, even control membranes displayed measurable ATPase activity. However, this activity was virtually insensitive to thapsigargin, a specific inhibitor of SERCA ATPases (41), while the additional ATPase activity of expressed wild type Ca²⁺-ATPase (WT assay), or that of an equivalent amount of native SR Ca²⁺-ATPase added to control membranes (SR + control membrane assay) was specifically inhibited by thapsigargin.

Specific Ca²⁺-ATPase activities were estimated for each mutant, after immunoquantification of the Ca²⁺-ATPase. The result is shown in Fig. 2B. As previously reported (42), a change of Pro-812 to alanine did not significantly affect the hydrolytic activity of Ca²⁺-ATPase, while cluster mutation P811A/P812A reduced this activity to about 50%. More pronounced effects were observed by changing the second couple of proline to alanine residues; the single mutation P821A reduced the ATPase activity by 50%, while mutation to alanine of the two proline residues 820 and 821 resulted in a Ca²⁺-ATPase with only 10% activity. Mutation to alanine of aspartic residues in loop 6–7 (ADA and AAA mutants) also reduced the ATPase activity to about 10% or less, as previously reported (32), confirming that these aspartic residues are important. Mutants E309Q and E771Q, previously shown to be defective in Ca²⁺-dependent control (26, 43), were also prepared as negative controls and found to be virtually devoid of ATPase activity.

**Fig. 3.** Ca²⁺ dependence of phosphoenzyme formation from [γ-³²P]ATP for aspartic and proline mutants of L6–7 loop, as measured by the filtration method. Incubation of 50 μg of total expressed protein (containing about 375 ng of SERCA, except for control membranes), with 2 μM [γ-³²P]ATP was performed as described under “Materials and Methods.” ³²P radioactivity bound to the acid-quenched proteins was counted after filtration and washing away the excess radioactivity. Results correspond to the mean values ± S.E. (n = 4). A, aspartic acid mutants ADA (open triangles pointing upward) and AAA (open triangles pointing downward) are compared with WT Ca²⁺-ATPase (open circles) and with control membranes (open squares). B, proline mutants P811A/P812A (closed diamonds), P821A (closed triangles pointing upward), and P820A/P821A (closed triangles pointing downward) are compared with wild type Ca²⁺-ATPase (open circles) and control membranes (open squares).

**Fig. 4.** Ca²⁺ dependence of phosphoenzyme formation from [γ-³²P]ATP for WT ATPase and ADA and P821A mutants, as measured after electrophoretic separation. Incubation of 50 μg of total proteins (containing about 375 ng of SERCA) with 2 μM [γ-³²P]ATP was performed at the indicated pCa, and radioactivity was quantified with a PhosphorImager after electrophoretic separation of a protein aliquot corresponding to about 150 ng of SERCA, as described under “Materials and Methods.” A, typical autoradiograms are shown for the WT ATPase and ADA mutant at various pCa values, as well as for native SR Ca²⁺-ATPase in the presence of 50 μg of bovine serum albumin at pCa 4. B and C, the Ca²⁺ dependence of phosphoenzyme formation from [γ-³²P]ATP of the ADA (open triangles pointing upward in B) and P821A (closed triangles pointing upward in C) mutated Ca²⁺-ATPases is compared with that of wild type ATPase (open circles) and control membranes (open squares in C). As a negative control, the result of an assay with mutant E309Q at pCa 4 (closed square) is also shown in C. Results correspond to the mean ± S.E. (n = 3).

Ca²⁺ Dependence of Phosphoenzyme Formation—Mutants were then tested for their ability to become phosphorylated from [γ-³²P]ATP, a reaction that requires binding of two Ca²⁺ ions to the protein. These experiments were first carried out by using a method based on filtration of perchloric acid-precipitated protein (Fig. 3) and were subsequently confirmed using a method based on electrophoretic separation of the phosphorylated intermediate (Fig. 4).

Fig. 3 shows the amount of protein phosphorylated from 2 μM [γ-³²P]ATP at various Ca²⁺ concentrations. A basal phosphorylation level of about 0.5 pmol/50 μg of protein was observed in all assays, including membranes of yeasts that did not express Ca²⁺-ATPase. This is attributable to phosphorylation of proteins other than Ca²⁺-ATPase in the membrane fraction. For WT Ca²⁺-ATPase, phosphorylation was distinctly dependent on pCa. The maximal level of Ca²⁺-dependent phosphorylation was about 1.5 pmol of phosphoenzyme/50 μg of total proteins, obtained for saturating Ca²⁺ concentrations (i.e., about 4 nmol of phosphoenzyme/mg of Ca²⁺-ATPase, corresponding to 0.45 mol of EP/mol Ca²⁺-ATPase, a reasonable value compared with results previously reported for intact SR (e.g., Refs. 11 and 16)). Activation by Ca²⁺ of phosphoenzyme formation occurred in two steps. The first step, in the micromolar range (10⁻⁷ – 10⁻⁴ M), corresponds to the high affinity binding of Ca²⁺ to the
ATPase. The subsequent increase in phosphorylation, observed in the millimolar range (10⁻³ – 10⁻² M) of Ca²⁺ concentrations, is probably due to the stabilization of phosphoenzyme arising from replacement of Mg²⁺ at the hydrolytic site by Ca²⁺ (see Refs. 45–48 and "Discussion").

Similar results were obtained when phosphoenzyme formation was measured after electrophoretic separation, as shown in Fig. 4. In that case, phosphorylated proteins contributing to the basal phosphorylation level were separated from phosphorylated Ca²⁺-ATPase. The autoradiograms displayed in Fig. 4A allow us to visualize phosphorylated wild type Ca²⁺-ATPase as a band migrating with an apparent molecular mass of about 100 kDa, like the native Ca²⁺-ATPase in SR (for reference, SR was added to control membranes at pCa 4; see right lanes in panel A); the amount of radioactivity in this region was very low at pCa 7, but it became prominent at higher free Ca²⁺ concentrations. Due to phosphoenzyme hydrolysis during electrophoretic separation, the maximal level of phosphorylated protein was lower than that measured in the filtration experiments, but radioactivity was retained to a significant extent, with a maximal value of 0.14 pmol of ³²P/150 ng of Ca²⁺-ATPase, i.e. close to 25% of the value before electrophoretic separation.

Similar experiments were performed with the Ca²⁺-ATPase mutants. Figs. 3A and 4A and B, show that the ADA and AAA mutants required much higher Ca²⁺ concentrations for phosphorylation than WT ATPase; at 10 m Ca²⁺, this resulted in full phosphorylation of the ADA mutant and partial phosphorylation of the AAA mutant. From these data, the apparent Ca²⁺ affinity for Ca²⁺ activation of the ADA mutant Ca²⁺-ATPase was estimated to be 2–5 m M. In contrast, proline mutants did not reveal any change in the apparent affinity for Ca²⁺ deduced from phosphoenzyme formation from [γ-³²P]ATP. This is shown for P811A/P812A and P820A/P821A in Fig. 3B, and for P821A in Figs. 3B and 4C. However, the maximum level of phosphoenzyme formed was significantly reduced for the cluster mutant P811A/P812A and even more for P820A/P821A. In the latter case, the effect of the mutation seems to be mainly related to the P821A mutation, since the residual Ca²⁺-dependent EP level was similar for P821A and for P820A/P821A. For the P812A mutant, phosphoenzyme formation was similar to that of WT ATPase at all pCa values (not shown). As a control for these measurements, phosphorylation from [γ-³²P]ATP of the E309Q mutant was also measured at pCa 4; as described in Ref. 26, no phosphorylation was observed (Fig. 4C).

Inhibition by Ca²⁺ of Phosphorylation from [³²P]Pi.—The filtration method used in Fig. 3 could not be used to measure phosphorylation from P₁, due to the fact that the P₁ concentration that had to be used (100 μM) resulted in a much larger background than in the experiments with [γ-³²P]ATP (2 μM ATP). Thus, phosphorylation from P₁ was quantified by our second method only, i.e. after separation on SDS-PAGE of the phosphorylated Ca²⁺-ATPase. The experiments were carried out both at pH 6.0 and 7.0, under conditions previously described (26–28, 49).

Fig. 5A shows examples of autoradiograms from dried gels after electrophoresis at pH 6.0. No signal was observed with control membranes. Panel B shows that the expressed wild type Ca²⁺-ATPase (open circles) was phosphorylated from P₁ in the absence of Ca²⁺ to the same extent as the equivalent amount (as deduced from Western blotting) of Ca²⁺-ATPase in native SR (closed circles); the maximal level of phosphorylation from P₁, obtained at low Ca²⁺ concentration, was in the same range as the maximal level of phosphorylation from ATP (see Fig. 4). As expected, the level of phosphorylated protein formed decreased as the Ca²⁺-free concentration increased; Ca²⁺ inhibited phosphorylation from P₁, with an apparent Ca²⁺ affinity of 40 μM under these conditions (pH 6.0, 20% Me₃SO). For the ADA mutant (open triangles in panel B), the maximal extent of phosphorylation from P₁ observed in the absence of Ca²⁺ was the same as that of the WT, but phosphorylation was much less sensitive to the presence of Ca²⁺, since the addition of 100 μM free Ca²⁺ was not sufficient to produce any inhibition; the K₅₀ value for Ca²⁺ inhibition of phosphorylation from P₁ of the ADA mutant was estimated to be around 4 mM. In contrast to these results, Fig. 5C shows that in the absence of Ca²⁺, the proline mutants P821A and P820A/P821A were phosphorylated to only 25% of the WT level, while the apparent affinity of these mutants for Ca²⁺ appeared not to be altered, as previously deduced also from the measurements of phosphorylation from ATP in Figs. 3B and 4C.

Inhibition by Ca²⁺ of Ca²⁺-ATPase phosphorylation from P₁ was also measured at pH 7.0, and the result is shown in Fig. 6. Fig. 6A displays results obtained for wild type and ADA mutated Ca²⁺-ATPases. It clearly appears that at pH 7.0 the curve for the ADA mutant is again strongly shifted toward high Ca²⁺.
concentrations, as compared with that of wild type Ca\(^{2+}\)-ATPase. For comparison, Fig. 6B shows the result of similar experiments performed with mutants E309Q and E771Q, previously characterized under the same conditions (27, 28). The E771Q mutant has a behavior that, from a phenomenological point of view, is similar to that of the ADA mutant, while the E309Q mutant, as previously found, has an apparent affinity for Ca\(^{2+}\) close to that of WT ATPase.

**Ca\(^{2+}\)-Dependent Pattern of Proteolysis by Proteinase K of Wild Type and Mutated Ca\(^{2+}\)-ATPases**—The above described phosphorylation experiments were supplemented with a completely different type of experiments, in which the ability of various mutants to bind Ca\(^{2+}\) was deduced from the Ca\(^{2+}\) dependence of their proteolysis pattern. Wild type Ca\(^{2+}\)-ATPase as well as E309Q, E771Q, and ADA mutants were submitted to proteolytic attack by proteinase K in the presence of various Ca\(^{2+}\) concentrations at neutral pH. The fragments were separated by SDS-PAGE, followed by Western blot. Among the fragments produced, peptides p95 and p83C (see their locations in Fig. 7D) were recognized by immunodetection with 577–588 Ab (Fig. 7A), and peptide p28N was recognized with 79B Ab (Fig. 7B). As illustrated in Fig. 7D and found previously for native Ca\(^{2+}\)-ATPase treated under similar conditions (31), p95 is produced by a proteolytic cleavage between residues Leu-119 and Lys-120, while p28N and p83C are both produced by a proteolytic cleavage between residues Thr-242 and Glu-243.

In Fig. 7A, the left lanes corresponding to proteinase K treatment of WT ATPase show that, at pCa 7, more than 50% of the Ca\(^{2+}\)-ATPase was proteolyzed, mainly leading to production of p95 peptide as well as a small amount of p83C (corresponding to 10% of starting material). Thus, the cleavage sites at Leu-119-Lys-120 and Thr-242-Glu-243 were both accessible, although cleavage at the former site was predominant. The presence of Ca\(^{2+}\) (lanes pCa 5.8 and 4) resulted in cleavage of about 70% of the Ca\(^{2+}\)-ATPase, with a modified cleavage pattern where p95 was no longer present, while p83C and p28N both accumulated to a higher extent. Open circles in Fig. 7C illustrate the Ca\(^{2+}\)-dependence of p28N formation in WT ATPase.

For the ADA mutated Ca\(^{2+}\)-ATPase (third group of lanes from the left in Fig. 7, panels A and B), proteinase K treatment at pCa 7 produced the same amount of p95 as for the wild type. However, at pCa 5.8, p95 was still detectable, while p83C and p28N were present to a lesser extent than in the wild type. At pCa 4, the amount of p28N formed remained lower than that of the wild type (see Fig. 7C, open triangles compared with open circles), while p95 disappeared completely and p83C started to accumulate. Thus, the apparent affinity with which Ca\(^{2+}\) modified the proteolytic pattern of the ADA mutant was reduced compared with that for WT ATPase, in agreement with the phosphorylation results in Figs. 3–7. Nevertheless, since in the absence of Ca\(^{2+}\) the ADA mutant has the same pattern of proteolysis as WT ATPase, the conformational state of the Ca\(^{2+}\)-free form of this mutated ATPase seems to be similar to that of WT ATPase.

The other groups of lanes in Fig. 7, A and B, show the proteolysis pattern for the E309Q and E771Q mutants. The behavior of the E309Q mutant was remarkable; at pCa 7, p95 formation was not observed at all, while distinct amounts of p28N (see Fig. 7C, closed squares) and p83C were present, suggesting a change in the proteolytic cleavage pattern in the absence of Ca\(^{2+}\). At higher Ca\(^{2+}\) concentrations, still larger amounts of p83C and p28N were present; as shown in Fig. 7C, the amount of p28N (closed squares) at pCa 4 was as high as that of WT (open circles). This indicates that the E309Q mutant has definitely retained sensitivity to the presence of Ca\(^{2+}\), in agreement with the phosphorylation results in Fig. 6B, but that its conformation in the Ca\(^{2+}\)-free state differs from that of WT ATPase. The situation was also remarkable with the E771Q mutated ATPase, which was more strongly proteolyzed than WT ATPase and the other mutants. At pCa 7, p28N was again present, in an amount equivalent to that for E309Q (open diamonds in Fig. 7C compared with closed squares). At higher pCa, p28N did not accumulate (open diamonds in Fig. 7); this was also the case for p83C, which even at higher Ca\(^{2+}\) levels was only present in a low amount. Again in agreement with the results of the phosphorylation experiments, this suggests that the mutated E771Q Ca\(^{2+}\)-ATPase is indeed only very poorly sensitive to the presence of Ca\(^{2+}\) (although fragments may be degraded too quickly under the conditions tested to detect a difference). In addition, for E771Q as for E309Q, the conformational state of the Ca\(^{2+}\)-free ATPase differs from that of WT ATPase.

**DISCUSSION**

Functional Properties of the L6–7 Mutants—The N-terminal side of the L6–7 loop in Ca\(^{2+}\)-ATPase (see Fig. 1) is characterized by the presence of three aspartic residues, surrounded by two couples of proline residues. An important outcome of our investigation is that cluster mutations of the aspartic residues shifted the Ca\(^{2+}\)-ATPase affinity for Ca\(^{2+}\) to much higher concentrations than those required to activate wild type ATPase; the \(K_{a}^{\mathrm{Ca}}\) for Ca\(^{2+}\) activation of phosphorylation from ATP of the D813A/D818A mutated ATPase was shifted from the micromolar to the millimolar range, while the maximal level of phos-
phosphorylation remained the same as that of wild type ATPase (Figs. 3 and 4). It can therefore be concluded that the ADA mutant has an intact phosphorylation site and can still bind two Ca$^{2+}$ ions, although at higher Ca$^{2+}$ concentrations than WT ATPase. In agreement with this, the double and triple mutants D813A/D818A and D813A/D815A/D818A only displayed a very low ATPase activity when they were tested at $p_{Ca} 4$, (Fig. 2). Note that the single mutant D815A gave a fully active ATPase, while mutation of D813A alone resulted in only a moderate loss of activity of 56%.2

An unexpected feature of our phosphorylation experiments was that Ca$^{2+}$ stimulation of phosphoenzyme formation from ATP by WT ATPase revealed two steps, separated by a plateau around $p_{Ca} 5.5–3.5$ (Figs. 3A and 4B). However, a comparable behavior has been observed previously for purified ATPase or leaky SR vesicles under similar conditions (see, for example, Fig. 7 in Ref. 45). Stimulation of phosphorylation by micromolar Ca$^{2+}$ is caused by binding of Ca$^{2+}$ to the high affinity transport sites, but saturation of these sites is not necessarily accompanied by complete phosphorylation, since the latter is dependent on the balance between the overall rates of phosphorylation and dephosphorylation. Increasing the Ca$^{2+}$ concentration to millimolar values slows down the rate at which phosphoenzyme is hydrolyzed, presumably because of substitution of Ca$^{2+}$ for Mg$^{2+}$ at the catalytic site, and thereby increases the steady state level of phosphorylation (see Refs. 45–48). Conceivably, for the ADA mutant, the same substitution of Ca$^{2+}$ for Mg$^{2+}$ could also contribute to the rise of the steady state $E_P$ level at millimolar Ca$^{2+}$ concentrations. Consequently, the “apparent” affinity for Ca$^{2+}$ of the ADA mutant deduced from the phosphorylation data probably represents a “mixed” affinity, partly reflecting the usual exchange of Mg$^{2+}$ for Ca$^{2+}$ at the ATPase catalytic site and partly reflecting the binding of Ca$^{2+}$ at the transport sites with reduced affinity.

Mutation of both couples of proline residues to alanine affected the Ca$^{2+}$-ATPase in different ways. For the P811A/P812A double mutant, the resulting Ca$^{2+}$-ATPase activity (Fig. 2) and the ability to become phosphorylated from ATP (Fig. 3B) were not drastically affected, in good agreement with similar data previously reported for proline residue 812 (42). This suggests that the proline residues of the first doublet are not essential for Ca$^{2+}$-ATPase function. On the other hand, proline

---

2 T. Menguy, unpublished results.

---

**Fig. 7. Ca$^{2+}$-dependent proteolysis by proteinase K of wild type and mutated Ca$^{2+}$-ATPases.** Proteolysis by proteinase K of 150 ng of expressed wild type Ca$^{2+}$-ATPase or E309Q, E771Q, or ADA mutants was carried out at three different $p_{Ca}$ values, 7, 5.8, or 4, as described under “Materials and Methods.” Control unproteolyzed sample (lanes) and proteolyzed samples (+ lanes) were loaded on an SDS-polyacrylamide gel and after migration were transferred to a PVDF membrane as described under “Materials and Methods.” A, Part of the PVDF membrane corresponding to the region of migration of 80–110-kDa peptides. In this region, Ca$^{2+}$-ATPase, p95 and p83C were immunodetected with the 577–588 antibody (31). When present, p95 and p83C are indicated by arrows. B, part of the PVDF membrane corresponding to the region of migration of 20–30-kDa peptides. In this region, p28N was immunodetected with the 79B antibody (40) and, when present, is indicated with an arrow. C, levels of p28N formed from wild type Ca$^{2+}$-ATPase (open circles) and ADA (open triangles), E309Q (closed squares), and E771Q (open diamonds) mutated Ca$^{2+}$-ATPases in the presence of various Ca$^{2+}$ concentrations during proteolysis. Values were obtained by quantification of the bands (Molecular Analyst) and are given in arbitrary units. D, topological model of the Ca$^{2+}$-ATPase where positions of mutations (closed circles), proteolytic cleavage sites between Leu-119 and Lys-120 and between Thr-242 and Glu-243 (double lines), and epitopes for 577–588 Ab (open rectangle, approximate location) have been indicated. Peptides p95, p83C, and p28N are represented at the bottom.
residues 820–821 seem to play a more important role; we found that the Ca\(^{2+}\)-ATPase activity at pCa 4 of the corresponding double alanine mutant was only 10% that of wild type ATPase (Fig. 2B), and maximal phosphorylation from ATP and P\(_i\) was low. Comparable effects were obtained after a single mutation of Pro-821 to alanine, suggesting that this residue is more critical than Pro-820. Note that Pro-821 is well conserved in all transport ATPases (5). Nevertheless, a clear difference from the effect of mutation of the aspartic residues in the L6–7 loop is that after mutations of proline residues the apparent affinity of the ATPase for Ca\(^{2+}\) was not modified, as judged from the Ca\(^{2+}\)-dependence of ATPase phosphorylation from either ATP or P\(_i\) (Figs. 3–5). This shows that the proline residues of loop L6–7 do not participate in the formation of the Ca\(^{2+}\)-binding site, even indirectly by structuring it. One possible explanation for the effects observed on \(V_{\text{max}}\) and \(E_{\text{P,max}}\) is that the mutation of Pro-821 stabilizes the nonphosphorylated \(E_c\) species of the ATPase and that the integrity of this residue may be required to overcome rate-limiting step(s) in the cycle, associated with the \(E_c/E_{\text{P}}\), conformational conversion.

**The Relationship between Phosphorylation and Ca\(^{2+}\) Binding**—Because of the above mentioned biphasic Ca\(^{2+}\) dependence of ATPase phosphorylation from ATP, the complementary experiments in which we tested the inhibition by Ca\(^{2+}\) of \(P_i\)-derived phosphorylation are of critical importance. In agreement with the low affinity for Ca\(^{2+}\) of the ADA mutant suggested by the ATP-derived phosphorylation experiments, we observed that in this mutant \(P_i\)-derived phosphorylation was also less sensitive to Ca\(^{2+}\) inhibition than in WT ATPase, both under acidic and under neutral conditions (Figs. 5A and 6A). Some of the “classical” mutations have been reported to make the ATPase less sensitive to Ca\(^{2+}\) irrespective of pH in \(P_i\)-derived phosphorylation experiments (e.g., the well known E771Q, T799A, D800N, and E908A mutations), while for another subset of the “classical” mutants (e.g., E309Q and N796A) Ca\(^{2+}\) inhibits \(P_i\)-derived phosphorylation at pH 7 with an affinity that is not very different from that of wild type ATPase (10, 28, 50) (it should be mentioned that in acid media, the behavior of the N796A mutant becomes different from that of the E309Q mutant (26, 51). Our results in Fig. 6B show that WT ATPase, which was also more degraded than the wild type under the same conditions. This suggests that the conformational state of these sites in the absence of Ca\(^{2+}\) can be assumed to reflect the conformational equilibrium between the various forms of Ca\(^{2+}\)-free ATPase. On the other hand, binding of Ca\(^{2+}\) results in masking of the most N-terminal proteolytic site (Lys-120) and a higher degree of exposure of the second proteolytic site (Glu-243) in the \(\beta\)-strand region.

The extension of this technique to the study of the degradative reactions with structurally related and catalytically inactive mutants of the ATPase will be the subject of a forthcoming publication. We note that the biphasic behavior of Ca\(^{2+}\)-dependent phosphorylation at neutral pH presumably have the first binding site intact, while mutants in which Ca\(^{2+}\) is unable to prevent \(P_i\)-derived phosphorylation have the first binding site altered by the mutation. Applying the same rationale to the results obtained with the ADA mutant would suggest that in the ADA mutant the initial steps in Ca\(^{2+}\) binding are more crucial than Pro-821, with a role of the L6–7 aspartic residues in controlling at least the first of the two Ca\(^{2+}\)-binding sites. Whether such control is exerted through the first or second mechanism described above is the next question to be addressed.

**Proteolytic Degradation of ATPase Mutants by Proteinase K**—In the absence of a highly resolved three-dimensional structure, mild proteolysis is a powerful tool with which to investigate the spatial organization of transport ATPases (29–31, 52). Since several conformational changes probably occur upon Ca\(^{2+}\) binding to the Ca\(^{2+}\)-ATPase, it was of interest to study how the relationship among these conformational changes in wild type and mutated Ca\(^{2+}\)-ATPase, and the dependence of these changes on pCa. In the absence of bound Ca\(^{2+}\), proteinase K predominantly cleaves Ca\(^{2+}\)-ATPase in SR vesicles at two previously identified sites (31). The most N-terminal one of these two sites produces a short N-terminal peptide as well as peptide p95 (Lys-120-COOH terminus), while the other site gives rise to p28N (Thr-242-NH\(_2\) terminus) and p83C (Glu-243-COOH terminus). The differential accessibility of these sites in the absence of Ca\(^{2+}\) can be attributed to the conformational equilibrium between the various forms of Ca\(^{2+}\)-free ATPase. On the other hand, binding of Ca\(^{2+}\) results in masking of the most N-terminal proteolytic site (Lys-120) and a higher degree of exposure of the second proteolytic site (Glu-243) in the \(\beta\)-strand region.
including the second possibility mentioned above. These findings clearly establish the status of the ADA version as a “pure” Ca\(^{2+}\) affinity mutant, which undergoes the same conformational changes as the wild type ATPase but with a reduced Ca\(^{2+}\) binding affinity.

An important outcome of the proteolysis assays carried out in the absence of calcium is that the two nonconservative substitutions Asp → Ala that have been introduced into the ADA mutant do not seem to alter the conformation of the Ca\(^{2+}\)-free ATPase, whereas the replacement of a single Glu by a Gln at Gly-808 and Asp-818 for site 2, as identified by Clarke et al. (26) and separated in two classes by Andersen (10). The two intramembranous Ca\(^{2+}\)-binding sites are assumed to have a side-by-side location (see also Ref. 9). Aspartic acid residues 813, 815, and 818 of the L6–7 loop are pointing toward the cytosol. In the E(nH\(^{+}\)) state the two Ca\(^{2+}\)-binding sites are occupied by protons; site 2 is not directly accessible, possibly because it is masked by the L6–7 loop. The first Ca\(^{2+}\) ion interacts with aspartic residues 813, 815, and 818 of the L6–7 loop, which are the most accessible residues at this step. This initial interaction favors binding of Ca\(^{2+}\) in a subsequent step to the first membranous site (site 1), after reorientation of the loop, leading to the E(Ca1) state. This reorientation results in an increased accessibility of Ca\(^{2+}\) for interaction with the critical residues at the second site (site 2) and/or optimization of the positions of these residues, and thus binding of Ca\(^{2+}\) to site 2. At the same time, the loop is locked in position to function as a gate for the first bound Ca\(^{2+}\). This results in an E(Ca\(^{2+}\)/Ca\(^{2+}\)) state from which Ca\(^{2+}\) bound to site 1 cannot be released unless Ca\(^{2+}\) bound to site 2 is removed. This model is a possible alternative to the “single file” model, designed to explain evidence of sequential Ca\(^{2+}\) dissociation from the ATPase sites (13, 14, 53). In the single file model, the two Ca\(^{2+}\) ions bind in a sequential manner on top of each other in an ATPase crevice, i.e. first at site 1 and thus at site 2. In the present model with Ca\(^{2+}\) sites side-by-side, the sequential nature of Ca\(^{2+}\) binding and dissociation is caused by changes in the position of the L6–7 loop.

Arguing somewhat against this possibility, however, are the facts that mutation of the proline residues, in contrast to the aspartic acid residues, did not change the apparent affinity of ATPase for Ca\(^{2+}\) and that mutation of the Pro-820-Pro-821 doublet was more deleterious than mutation of the Pro-811-Pro-812 doublet, although the latter might have been expected to influence to a larger extent the conformation of the nearby M6 segment. In addition, as discussed above, the proteolysis experiments displayed in Fig. 7 allow us to conclude that in the absence of calcium the conformation of the ADA mutant is similar to that of WT ATPase.

Thus, as an alternative model, the L6–7 loop could be a direct contributor in the control of Ca\(^{2+}\) binding to Ca\(^{2+}\)-ATPase, as deduced from (a) our previous demonstration that Ca\(^{2+}\) has some affinity for the Gly-808-Gly-994 peptide but not for the shorter Asp-818-Gly-994 peptide (p20 and p19, respectively; Ref. 32) and (b) the present data that show that the removal of some of the oxygen atoms (Asp → Ala) located in the region from Gly-808 to Asp-818 of the L6–7 loop decreases the affinity for calcium by almost 3 orders of magnitude. Taking into account the recent proposal of a side-by-side location of the two Ca\(^{2+}\)-binding sites (9) and the cytosolic location of the L6–7 loop, Fig. 8 depicts a possible mechanism by which the aspartic residues of the L6–7 loop could participate in the initial steps of high affinity binding of Ca\(^{2+}\) to Ca\(^{2+}\)-ATPase; interaction of Ca\(^{2+}\) with the L6–7 loop could direct the first
Ca\(^{2+}\) ion toward the membrane-embedded liganding residues of the first site and open the way for binding of Ca\(^{2+}\) at the second site. In this model the sequential order during cytosolic Ca\(^{2+}\) dissociation is maintained by the L6–7 loop functioning as a cytosolic gate that prevents Ca\(^{2+}\) dissociation of the first bound Ca\(^{2+}\) before dissociation of the second bound Ca\(^{2+}\). In view of the emerging structure of the Ca\(^{2+}\)-ATPase transmembrane domain (7), it is not impossible that the L6–7 loop plays some kind of gating role at the entrance of the crevice leading to site 1.

Acknowledgments—We thank Anne-Marie Lompré (Université Paris Sud, Orsay) for the gift of the 7B9 antibody, Drs. D. Funpon and P. Urban (CNRS, Gif sur Yvette) for the gift of the yeast strain and the vector; and Adrienne Gomez de Gracia for technical assistance. We are grateful to Stéphanie Soulié and Drs. Francisco Centeno, Béatrice de Foresta, and Birte Juul for critical reading of the manuscript.

REFERENCES

1. de Meis, L., and Vianna, A. L. (1979) Annu. Rev. Biochem. 48, 275–292
2. De Pont, J. J. H. M., and Boning, S. L. (1981) in Membrane Transport (Bonington, S. L., and de Pont, J. J. H. M., eds) pp. 209–234, Elsevier North-Holland Biomedical Press, Amsterdam, Holland
3. Skou, J. C., and Esman, M. (1992) J. Bioenerg. Biomembr. 24, 249–271
4. Inesi, G., and Kurzmack, M. (1980) J. Biol. Chem. 255, 15931–15946
5. Fujimori, T., and Jencks, W. P. (1992) J. Biol. Chem. 267, 1111–1123
6. Orlowski, S., and Champeil, P. (1991) J. Biol. Chem. 267, 1329–1342
7. Juul, B., Turc, H., Durand, M. L., Gomez de Gracia, A., Denoroy, L., Keller, J. V., Chameil, P., and le Maire, M. (1995) J. Biol. Chem. 278, 20123–20134
8. Falsone, P., Mgluy, T., Corre, P., Bouneau, L., Gomez de Gracia, A., Soulié, S., Centeno, F., Mallia, A., Kamchik, P., and le Maire, M. (1997) J. Biol. Chem. 272, 17255–17262
9. Swarts, H. G., Rlaaassen, C. H., Boer, M., Fransen, J. A., De Pont, J. J. H. M. (1996) J. Biol. Chem. 271, 29764–29772
10. Centeno, F., Deschamps, S., Lopere, A. M., Anger, M., Moutin, M. J., Dupont, Y., Palmgren, M. G., Villalba, J. M., Keller, J. V., Falsone, P., and le Maire, M. (1994) FEBS Lett. 354, 117–122
11. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, M. D., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 76–85
12. Lacapère, J. J., Gouille, M. P., Champeil, P., Guillain, J. A. (1981) J. Biol. Chem. 256, 2302–2306
13. Combettes, L., Claret, M., and Champeil, P. (1993) Cell Calcium 14, 279–292
14. Inesi, G. (1981) Biochim. Biophys. Acta 658, 1–51
15. Brandl, C. J., Green, N. M., Korczak, B., and MacLennan, D. H. (1986) Cell 44, 597–607
16. Zhang, P., Toyoshima, C., Yonekura, K., Green, N. M., and Stokes, D. L. (1998) Nature 392, 835–839
17. Möller, J. V., Ning, G., Maunsbach, A. B., Fujimoto, K., Asai, K., Juul, B., Lee, Y.-J., Gomez de Gracia, A., Falsone, P., and le Maire, M. (1997) J. Biol. Chem. 272, 29015–29032
18. MacLennan, D. H., Rice, W. J., and Green, N. M. (1997) J. Biol. Chem. 272, 28815–28818
19. Andersen, J. P. (1995) Biosci. Rep. 15, 243–261
20. Inesi, G., Kurzmack, M., Coan, C., and Lewis, D. E. (1980) J. Biol. Chem. 255, 3025–3033
21. Petichory, J. R., and Jencks, W. P. (1992) J. Biol. Chem. 267, 18475–18487
22. Petichory, J. R., and Jencks, W. P. (1988) Biochemistry 27, 5553–5564
23. Inesi, G. (1987) J. Biol. Chem. 262, 16338–16342
24. Orlovski, S., and Champeil, P. (1991) Biochemistry 30, 352–361
25. Orlovski, S., and Champeil, P. (1991) Biochemistry 30, 352–361
26. Dupont, Y. (1980) Eur. J. Biochem. 109, 231–238
27. Takanawa, H., and Makino, M. (1981) Nature 290, 271–273
28. Watanabe, T., Lewis, D., Nakamoto, R., Kurzman, M., Fronticelli, C., and Inesi, G. (1981) Biochemistry 20, 6671–6675
29. Hanel, A. M., and Jencks, W. P. (1990) Biochemistry 30, 11320–11330
30. Orlovski, S., and Champeil, P. (1993) Biochemistry 30, 11331–11342
31. Yamaguchi, M., and Kanazawa, T. (1985) J. Biol. Chem. 260, 4896–4900
32. Levy, D., Seigneuron, M., Bluzat, A., and Rigaud, J.-L. (1990) J. Biol. Chem. 265, 19524–19534
33. Rice, W. J., and MacLennan, D. H. (1996) J. Biol. Chem. 271, 31412–31419
34. McIntosh, D. B. (1998) in Advances in Molecular and Cell Biology (Bittar, E. Edward, and Anderson, J. P., eds) pp. 33–99, JAI Press Inc., London
35. Clarke, D. M., Muraizama, K., Loo, T. W., Leberer, E., Inesi, G., and MacLennan, D. H. (1989) J. Biol. Chem. 264, 11246–11251
36. Clarke, D. M., Loo, T. W., Inesi, G. and MacLennan, D. H. (1989) Nature 339, 476–478
37. Clarke, D. M., Loo, T. W., Inesi, G. and MacLennan, D. H. (1989) Nature 339, 476–478
38. Clarke, D. M., Loo, T. W., Inesi, G. and MacLennan, D. H. (1989) Nature 339, 476–478
39. Clarke, D. M., Loo, T. W., Inesi, G. and MacLennan, D. H. (1989) Nature 339, 476–478
40. Clarke, D. M., Loo, T. W., Inesi, G. and MacLennan, D. H. (1989) Nature 339, 476–478
41. Clarke, D. M., Loo, T. W., Inesi, G. and MacLennan, D. H. (1989) Nature 339, 476–478
42. Clarke, D. M., Loo, T. W., Inesi, G. and MacLennan, D. H. (1989) Nature 339, 476–478
43. Clarke, D. M., Loo, T. W., Inesi, G. and MacLennan, D. H. (1989) Nature 339, 476–478