# Regulation of the Calcium Ion Pump of Sarcoplasmic Reticulum: Reversible Inhibition by Phospholamban and by the Calmodulin Binding Domain of the Plasma Membrane Calcium Ion Pump†

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ABSTRACT: A 45 amino acid peptide (A45) corresponding to the phospholamban (PLN) binding domain of the sarcoplasmic reticulum (SR) ATPase was synthesized. Circular dichroism experiments have shown that the peptide had a predominantly random-coil conformation but adopted a higher proportion of secondary structure in the presence of a synthetic 32 amino acid peptide corresponding to the hydrophilic portion of PLN. A similar conformational change was induced by the synthetic calmodulin binding domain of the plasma membrane $Ca^{2+}$ pump (peptide C28W), which acts as an endogenous inhibitor of the pump and is homologous to PLN. Cross-linking experiments have shown that peptide C28W interacted with peptide A45. The $Ca^{2+}$-pumping activity of cardiac SR, which contains endogenous PLN, was stimulated about 30% by peptide A45. The stimulation was maximal at submicromolar $Ca^{2+}$ levels and tended to disappear at higher $Ca^{2+}$ concentrations. By contrast, the $Ca^{2+}$-pumping activity of skeletal muscle SR, which lacks endogenous PLN, was unaffected. Peptide C28W strongly inhibited the pumping activity of skeletal muscle SR, and peptide A45 reversed the inhibition. The results suggest that peptide A45 competed with the ATPase for phospholamban or for peptide C28W, removing the inhibition of the pump. Thus, the exogenous inhibitor of the SR $Ca^{2+}$-ATPase, PLN, and the internal inhibitor of the plasma membrane $Ca^{2+}$-ATPase, peptide C28W, are functionally analogous.

Slow-twitch muscles express an isoform of the sarcoplasmic reticulum (SR) $Ca^{2+}$-ATPase (Brandl et al., 1987), whose activity is regulated by the endogenous protein phospholamban (PLN) (Tada et al., 1975), for which cDNAs have been cloned and sequenced (Fujii et al., 1987). PLN is the substrate of various protein kinases, and its phosphorylation leads to the stimulation of the ATPase (Movsesian et al., 1984; Le Peuch et al., 1979). It is an amphiphilic molecule of 52 amino acids, probably anchored to the membrane by the highly hydrophobic C-terminal domain. The hydrophilic cytosolic N-terminal portion contains the phosphorylation sites and several positively charged residues which confer an evident alkaline character to the unphosphorylated protein. The cytosolic portion of the molecule can be viewed as an amphipathic $\alpha$ helix and is structurally related to the autoinhibitory calmodulin binding domain of the $Ca^{2+}$-pumping ATPases of the plasma membrane. Compelling evidence has shown that nonphosphorylated PLN is an inhibitor of the SR $Ca^{2+}$-ATPase. Reconstitution studies have shown inhibition upon addition of PLN (Inui et al., 1986) or of its cytosolic portion (Kim et al., 1990). Inhibition is also induced by synthetic peptides deriving from the autoinhibitory calmodulin binding domain of the plasma membrane $Ca^{2+}$ pump (Chiesi et al., 1991). Both PLN and these peptides enrich the surface of the SR membrane in fixed positive charges, which inhibit $Ca^{2+}$ transport (Xu & Kirchberger, 1989; Chiesi & Schwaller, 1989). Phosphorylation of PLN (or of its synthetic cytosolic portion) reverses the change in surface charge and thus the inhibition of transport. Cross-linking experiments using a photoactivatable derivative of PLN have recently shown that purified PLN and the cardiac SR ATPase indeed interact in the solubilized state (James et al., 1989). The domain of the ATPase involved in the interaction has been identified C-terminally to the aspartate (Asp-351), which becomes phosphorylated during the transport cycle, in a region surrounding two Lys residues.

The present investigation was undertaken to verify two points: first, that the domain of the ATPase to which the photoactivatable PLN derivative becomes conjugated is actually directly involved in the formation of the native PLN binding site and, second, that the site of interaction is involved in the regulation (inhibition) of the pump. The putative PLN binding domain of the cardiac SR $Ca^{2+}$-ATPase has been synthesized, and its ability to interact with PLN and to reverse the inhibition of the ATPase has been investigated. Analogous experiments with the synthetic autoinhibitory calmodulin binding domain of the plasma membrane $Ca^{2+}$-pump, which is structurally homologous to PLN, have yielded similar results.

## Experimental Procedures

*Materials.* $^{45}CaCl_2$ was obtained from New England Nuclear. MOPS[1] and EGTA were purchased from Sigma Chemical Co. (St. Louis, MO). For peptide synthesis, amino acid analysis, and sequencing, the products of Applied Biosystems (Foster City, CA) were used. All other reagents were

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[1] Abbreviations: Boc, *tert*-butoxycarbonyl; CD, circular dichroism; DCCD, dicyclohexylcarbodiimide; DTT, dithiothreitol; DVB, divinylbenzene; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MOPS, morpholinopropanesulfonic acid; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PTC, phenylthiocarbamyl; PTH, phenylthiohydantoin; tBu, *tert*-butyl; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

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Table I: Sequences of the Synthetic Peptides[a]

| | |
|---|---|
| | PLN Binding Domain of the SR $Ca^{2+}$-ATPase |
| A12 | V-K-C-H-Q-T-D-G-L-V-E-L |
| A21 | G-E-V-H-K-D-D-K-P-V-K-C-H-Q-T-D-G-L-V-E-L |
| A45 | L-K-V-D-G-E-T-C-S-L-N-E-F-T-I-T-G-S-T-Y-A-P-I-G-E-V-H-K-D-D-K-P-V-K-C-H-Q-T-D-G-L-V-E-L |
| | Hydrophilic Portion of PLN |
| P12 | R-S-A-I-R-R-A-S-T-I-E-M |
| P20 | Ac-M-D-K-V-Q-Y-L-T-R-S-A-I-R-R-A-S-T-I-E-M |
| P32 | Ac-M-D-K-V-Q-Y-L-T-R-S-A-I-R-R-A-S-T-I-E-M-P-Q-Q-A-R-Q-N-L-Q-N-L-F-$NH_2$ |
| | Autoinhibitory Calmodulin Binding Domain of the Plasma Membrane $Ca^{2+}$-ATPase |
| C28W | L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-S |
| C28W* | ($^3$H)Ac-L-R-R-G-Q-I-L-W-F*-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-S |

[a] C28W* is the derivatized peptide containing an (azitrifluoroethyl)phenylalanine in position 9. The synthesis procedures are described in the Experimental Procedures section.

of the highest purity grade available and were purchased from Fluka AG (Buchs, Switzerland).

*Biological Material.* SR membranes were isolated from rabbit white muscle and from dog heart as previously described [Inesi et al. (1973) and Chamberlain et al. (1983), respectively]. The intermediate microsomal fraction, consisting mainly of elements deriving from longitudinal and cisternal elements of the SR, was utilized. Membranes were stored until used in 300 mM sucrose/10 mM MOPS, pH 7, at −70 °C.

*Synthesis of Peptides C28W, C28W*, and P32.* The sequences of the synthetic peptides used are summarized in Table I. Details of the synthesis, deprotection, and purification of peptides C28W (L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-S) and C28W* ([$^3$H]Ac-L-R-R-G-Q-I-L-W-F*-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-S) are described in Vorherr et al. (1990) and Falchetto et al. (1991), respectively. The asterisk denotes the (azitrifluoroethyl)-phenylalanine in position 9. The synthesis of peptide P32 (Ac-M-D-K-V-Q-Y-L-T-R-S-A-I-R-R-A-S-T-I-E-M-P-Q-Q-A-R-Q-N-L-Q-N-L-F-$NH_2$) is described in Chiesi et al. (1991). The peptides were synthesized on an Applied Biosystems peptide synthesizer Model 431 using the Fmoc/tBu strategy with 1-methyl-2-pyrrolidone for coupling and washing according to the standard protocol for the synthesizer using DCCD/HOBt activation. The first amino acid was attached according to the cycle for loading the first amino acid onto the (hydroxymethyl)phenoxypolystyrene resin (1.0 mmol/g, 1% DVB). The peptide was synthesized using Pmc protection for the Arg residues and trityl protection for the Cys and His residues.

*Synthesis of Peptides P12 and P20.* A 6.2- and a 16-fold excess of Fmoc-amino acid derivative was used for the synthesis of P12 and for P20, respectively. Complete acetylation of the side-chain-protected peptide resin P20 was achieved with 5 mL of a mixture of acetic anhydride, pyridine, and methylene chloride (1:1:8). Cleavage of the peptides P12 and P20 was performed in 3 mL of 95% TFA/1% ethanedithiol/2% thioanisole/2% methyl sulfide for 2.0 h at room temperature. A total of 64.8 mg of crude product was obtained from 152 mg of peptide resin after precipitation and washing with ethyl ether in the case of peptide P12. A total of 24.5 mg of purified peptide was obtained from 50 mg of crude peptide after preparative HPLC using buffers A and B (see below). In the case of peptide P20, 51.6 mg of crude product was obtained from 107 mg of acetylated peptide resin. A total of 22.4 mg of purified peptide was obtained from 41 mg of crude peptide after preparative HPLC using buffers A and B. Analytical HPLC was performed on a 30 × 2.1 mm $C_8$ column using buffers A and B at a flow rate of 1 mL/min. The amino acid analysis agreed with the expected ratios.

*Synthesis of Peptides A12, A21, and A45.* A 5.9- and a 10-fold excess of Fmoc-amino acid derivative was used for the synthesis of peptides A12 and A21, respectively. Cleavage of peptides A12 and A21 was performed in 3 mL of 95% TFA/1% ethanedithiol/2% thioanisole/2% methyl sulfide for 2.5 h at room temperature. A total of 41.8 mg of crude product was obtained from 82 mg of peptide resin after precipitation and washing with ethyl ether in the case of peptide A12. A total of 17.3 mg of purified peptide was obtained from 25.1 mg of crude peptide after preparative HPLC using buffers A and B. In the case of peptide A21, 144.2 mg of crude product was obtained from 240 mg of peptide resin. A total of 56 mg of purified peptide A21 was obtained from 78.4 mg of crude peptide after preparative HPLC using buffers A and B.

The synthesis of peptide A45 was achieved by continuing the synthesis proocedure with the fully protected peptide resin of peptide A21. A 10-fold excess of amino acid derivative was used, and capping with acetic anhydride was performed according to the instructions in the manual for the small-scale Fmoc modules. The same cleavage mixture used for peptides A12 and A21 was employed for 2 h at room temperature for the cleavage reaction of peptide A45. A total of 112 mg of crude product was obtained from 165 mg of peptide resin after precipitation and washing with ethyl ether. A total of 7.7 mg of purified peptide was obtained from 87 mg of crude peptide after preparative HPLC using buffers A and B and rerunning the sampled fractions under the same conditions.

The characterization of the purified peptides was performed by analytical HPLC using either buffers A and B (peptides A12 and A21) or C and D (peptide A45) and amino acid analysis. In addition, mass spectrometry analysis was performed on a Bio ION time of flight instrument for peptide A45. The mass of the $MH^+$ peak was 4905.4 (the mass calculated according to the sequence was 4904.4), and no other peaks were detected. The sequence of the 19 N-terminal amino acids of peptide A45 was confirmed after the cross-linking experiment.

*HPLC Analysis and Sequencing of the Purified Peptides.* Semipreparative HPLC was carried out using Nucleosil reversed-phase material packed in a Macherey and Nagel column (Oensingen, Switzerland). The reversed-phase buffers were (A) 0.1% TFA in water and (B) 0.05% TFA/50% *n*-propanol in water. The peptides were purified on a 250 × 55 mm $C_8$ column (10 μm, 300 Å) on a linear or stepwise linear gradient from A to B. Analytical control for peptides A12 and A21 was performed in the same buffer system using a 2.1 × 30 mm $C_8$ column (Aquapore 7 μm, 300 Å) obtained from Applied Biosystems.

Analytical control for peptide A45 was performed on a 100 × 2 mm $C_4$ column (Aquapore 7 μm, 300 Å) obtained from Applied Biosystems in the following buffer system: (C) 0.1% TFA in water; (D) 0.1% TFA/70% acetonitrile in water. The photo-cross-linked products were separated on a Nucleosil $C_{18}$ (Macherey and Nagel) 60 × 4 mm, 3-μm, 100-Å column. One-minute fractions were collected. Radioactivity was measured in Ready Safe liquid scintillation cocktail (Beckman Instruments Inc., Fullerton, CA) with a Beckman LS 1801 counter. HPLC chromatography was carried out using Ap-

plied Biosystems and LKB equipment (LKB, Uppsala, Sweden). UV detection was performed at 210 nm.

An Applied Biosystems Derivatizer 420A with on-line PTC detection with the Model 130A Applied Biosystems analyzer was used for derivatization, separation, and identification of the amino acids. Amino acid analysis was carried out for all synthetic peptides. Sequencing was carried out using an Applied Biosystems 470A sequencer with 120A on-line PTH detection.

*Circular Dichroism Experiments.* The CD spectra of peptides P32, C28W, and A45 and peptide mixtures in solution at pH 6.0 (10% TFE) and pH 8.5 (20% TFE) were obtained using a Jasco J-600 spectropolarimeter connected to an IBM-AT computer (Eastern, MD). The solutions were clarified by centrifugation, and the peptide concentrations were determined by amino acid analysis. The CD data were recorded in a 0.2- or a 0.1-mm quartz cell at 23 °C. The spectra were run at a scanning speed of 20 nm/min and a time constant of 1.0 s over the wavelength range 190–250 nm. One scan was performed for each spectrum. The spectra were corrected for spurious signals generated by the solvent and smoothed by digital filtering according to the instructions of the manufacturer. Mathematically added CD spectra were calculated after background correction and smoothing of the appropriate spectra. In the experiments on the peptide complexes, mean residue ellipticities were calculated on the basis of the sum of the peptide concentrations and the average molecular weight of all residues.

*Photocoupling of the C28W* Peptide to the Phospholamban Binding Domain (A45).* The concentration of peptide A45 in 10 mM MOPS buffer, pH 7.5, was 190 $\mu$M, and peptide C28W* was added to a final concentration of 60 $\mu$M (total volume 260 $\mu$L). DTT was added to a final concentration of 5 mM as a protective agent against light-induced oxidation and to prevent unspecific reactions of the carbene generated from the diazirine group. Photolysis was carried out for 1.5 min with the 450-W lamp of a SPEX Fluorolog 1680 (Metuchen, NJ).

*Analysis of the Cross-Linked Peptides and Digestion with the V8 Protease.* The photo-cross-linked product was separated on a $C_{18}$ (Macherey and Nagel) HPLC column using buffers C and D. The fractions were counted, and aliquots of the radioactive fractions were sequenced. The radioactive fractions corresponding to the free acetylated C28W* peptide and to the cross-linked product were pooled, the acetonitrile was removed under a stream of nitrogen, and the fractions were digested with V8 protease. A total of 1 $\mu$g of protease/9 $\mu$g of peptide was used for the digestion experiments in 40 mM ammonium acetate, pH 4.0, containing 5% acetonitrile. The digestion was carried out for 24 h at 37 °C. The digest was analyzed again by HPLC, and the main radioactive fraction was subjected to sequencing.

*$Ca^{2+}$-Uptake Measurements.* The rate of $Ca^{2+}$ uptake by the SR membranes was measured at 37 °C using the Millipore filtration technique and $^{45}Ca^{2+}$. SR membranes, at a concentration ranging from 10 to 50 $\mu$g/mL, were incubated in 100 mM KCl/300 mM sucrose/5 mM $MgCl_2$/50 mM MOPS, pH 7.0/5 mM $NaN_3$/3 mM potassium oxalate, and 0.5 mM EGTA. $CaCl_2$ was added at various concentrations, and $Ca_{free}$ was calculated according to Fabiato and Fabiato (1979). When required, the synthetic peptides were preincubated with the SR membranes in the uptake medium. After 10 min of preincubation, 2 mM ATP was added to start the uptake reaction. The rate of uptake was determined from the linear portion of the reaction which ranged between 2 and 5 min after the addition of ATP, depending on the free $Ca^{2+}$ concentration.

## Results

*Circular Dichroism Studies of the Complex between Peptide A45, the Hydrophilic Portion of PLN, and Peptide C28W.* The studies were performed in 10 mM phosphate buffer, pH 6, containing 10% TFE in the case of peptide C28W, which is poorly soluble in aqueous solutions, and in 5 mM phosphate buffer, pH 8.5, containing 10 or 20% TFE in the case of peptide P32. In the case of the latter peptide, a significant content of $\alpha$ helix was observed only using 20% TFE in aqueous buffer. These conditions proved essential to monitor complex formation, since one of the components (P32 or C28W) had already adopted a high proportion of ordered secondary structure while the CD spectrum of peptide A45 still showed a predominant random-coil conformation.

The spectrum of peptide A45 in phosphate buffer showed no significant content of secondary structure between pH 5 and 8.5. A broad single minimum at 203–204 nm, typical of a random-coil conformation, appeared in this pH range. No significant spectral changes were observed in the presence of 100 mM NaCl; however, the spectrum became clearly helical in the presence of 35% TFE. In this case the minimum shifted to 209 nm and a zero cut of the $x$-axis was seen at 200 nm (data not shown).

Peptide P32 also showed a predominant random-coil conformation in aqueous buffer solutions. However, in this case 20% TFE induced a higher helical content as compared to that in the CD spectrum of peptide A45. The minimum was at 212 nm, and the zero cut of the $x$-axis was at 204 nm (Figure 1A). Under these conditions the spectrum of peptide A45 still displayed random-coil properties, although the ellipticities at 220 nm had increased (Figure 1A). Upon mixing of the two peptides, the observed flattening of the spectrum of P32 indicated interaction. The random-coil contribution of peptide A45 to the calculated spectrum of the complex was higher than that measured for the peptide mixture (Figure 1B), indicating that secondary structure had been induced by the complex formation.

When peptide A45 and the autoinhibitory calmodulin-binding peptide C28W were mixed in a 10% TFE solution, a qualitatively similar, but more pronounced, effect was observed (Figure 1D): the minimum in the CD spectrum of peptide A45 remained at about 203 nm, with contributions of $\alpha$ helix and/or $\beta$ sheet structure explaining the negative ellipticities at about 220 nm (Figure 1C). As reported earlier (Vorherr et al., 1990), the spectrum of peptide C28W showed features of $\alpha$ helix and $\beta$ sheet (Figure 1C). Most of the random-coil portion of peptide A45 adopted secondary structure in the peptide mixture when compared to the spectrum calculated by adding the CD spectra of the single components. Also in this case, the measured ellipticity of the complex was much lower than theoretically expected for a mixture of the two peptides. In both cases, the higher proportion of ordered secondary structure in the complexes can be attributed to the induction of ordered secondary structure in peptide A45, since peptide P32 already contained a significant portion of secondary structure. In the case of the peptide mixture P32–A45, a precipitate was observed at higher concentrations when TFE was added to a final concentration of 10%. These observations show that peptide A45 indeed forms complexes with the inhibitory peptides, albeit only at relatively high concentrations.

*Cross-Linking of Peptide A45 to the Photoactivatable Peptide C28W*.* The photoactivatable peptide C28W*,

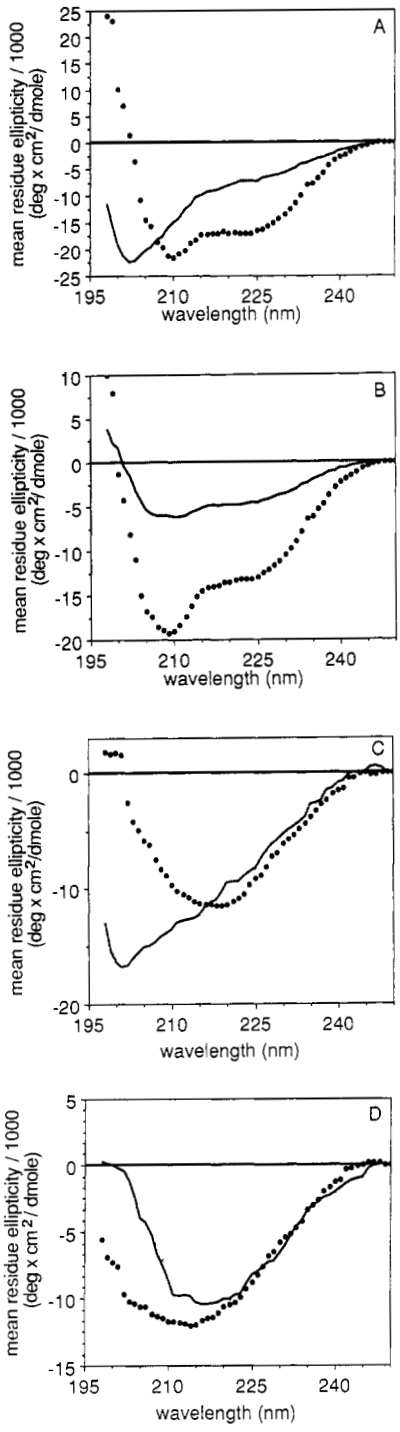


FIGURE 1: CD spectra of the peptides and peptide complexes. (A) CD spectra in 5 mM phosphate buffer, pH 8.5, containing 20% TFE. The figure shows peptides P32 (●) and A45 (—). The peptide concentrations were 3.2 mg/mL for P32 and 1.9 mg/mL for A45. (B) CD spectra of the peptide complex (peptides A45 and P32) calculated according to the spectra of the single components (A45 and P32, part A) (●) and the measured peptide mixture (—). The peptide concentrations were 1.6 mg/mL for P32 and 0.95 mg/mL for A45. (C) CD spectra of peptides C28W (●) and A45 (—) in 5 mM phosphate buffer, pH 6.0, containing 10% TFE. The peptide concentrations were 0.33 mg/mL for C28W and 0.3 mg/mL of A45. (D) CD spectra of the peptide complex calculated according to the spectra of the single components (A45 and C28W) (●) and the measured peptide mixture (—). The peptide concentrations were 0.17 mg/mL for C28W and 0.15 mg/mL for A45. The spectra were recorded as described in the Experimental Procedures section.

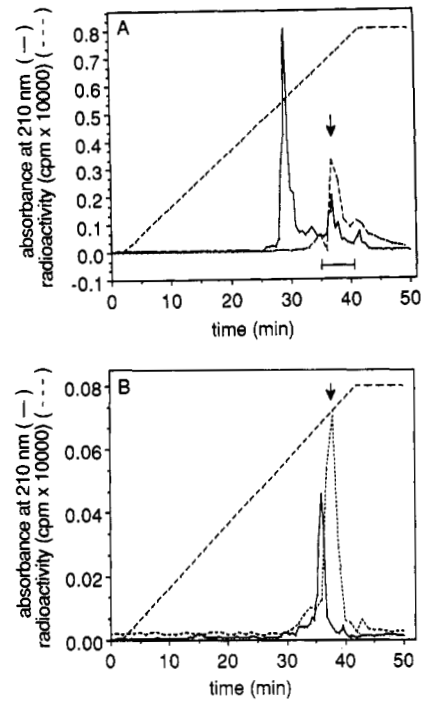


FIGURE 2: (A) HPLC trace of peptides A45 and C28W* after photo-cross-linking. The early eluting main peak corresponds to peptide A45. The peptide mixture was separated on a $C_{18}$ reversed-phase column (Nucleosil, 3 μm, 100 Å, 80 × 4 mm). The flow rate was 300 μL/min, detection was at 210 nm (—), and 5-μL aliquots were withdrawn for scintillation counting (- - -). The gradient using buffers C and D is indicated with a dashed line (2 min 0% B, 42 min 100% B). The solid bar shows the fractions pooled for digestion with the V8 protease. (B) HPLC trace after V8 digestion of the radioactive fractions shown in panel A. The pool of radioactive fractions from the HPLC run shown in panel A was loaded onto the C18 column (see panel A) and run by applying the same gradient. Additional details are found in the Experimental Procedures section. The fraction containing most of the radioactivity, indicated by an arrow, was submitted to sequence analysis.

carrying a $^3$H-labeled acetyl group at the N-terminus, was incubated with peptide A45 and cross-linked to it by photoactivation in the presence of 5 mM DTT (see the Experimental Procedures section). The concentrations of peptide A45 and peptide C28W* were 190 and 60 μM, respectively. After photocoupling, the sample was run on a reversed-phase $C_{18}$ HPLC column, the fractions were sampled, and their radioactivity was counted (Figure 2A). The fraction corresponding to the peak marked with an arrow was submitted to amino acid sequencing: the first 19 amino acids could be read unambiguously, showing that the sequence corresponded to the N-terminal domain of peptide A45. The fractions indicated with a bar containing free peptide C28W* and the cross-linked product were sampled and digested with V8 protease to identify more precisely the site of interaction (the protease did not attack the C28W* moiety of the cross-linked product). After digestion, the mixture was rerun on a $C_{18}$ column (Figure 2B) and the main radioactive peak was submitted to sequencing. Two sequences could be read unambiguously, the major one (V-I-L-P-N-N-D) corresponding to the N-terminal domain of the protease accidentally coeluting with the labeled peptide. The second sequence (V-X-K-D-D) corresponded to the C-terminal domain of peptide A45. A control digestion of unlabeled peptide A45 showed that no fragments produced by proteolysis eluted in this region. The experiment thus showed that a complex between peptides C28W* and A45 had been formed and that the Phe in position 9 of peptide C28W* had interacted with the C-terminal domain of peptide A45.

*Effect of Synthetic SR ATPase Peptides on the $Ca^{2+}$ Transport Activity of Cardiac and Skeletal Muscle SR Membranes.* The ATPase peptides used (A12, A21, A45)

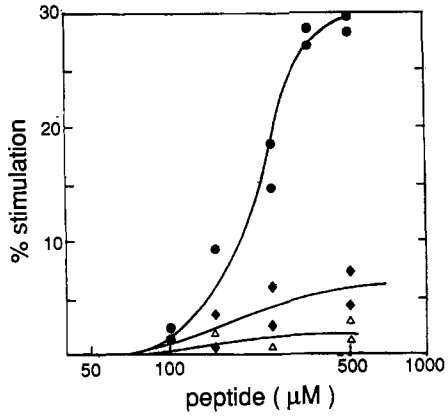


FIGURE 3: Effect of SR $Ca^{2+}$-ATPase peptides A12, A21, and A45 on the $Ca^{2+}$-uptake activity of cardiac SR membranes. Cardiac SR vesicles were incubated at 37 °C in the presence of various amounts of synthetic peptides A12 (△), A21 (◆) or A45 (●), and $Ca^{2+}$ uptake was measured as described in the Experimental Procedures section. The free $Ca^{2+}$ concentration in the uptake medium was maintained at 0.3 μM with an appropriate EGTA buffer. $Ca^{2+}$ uptake was linear for about 5 min, and the percentage of stimulation of the initial uptake was calculated. The control uptake rate (i.e., the rate corresponding to 0% stimulation) was 140 nmol $min^{-1}$ $mg^{-1}$. The data points given refer to two independent experiments.

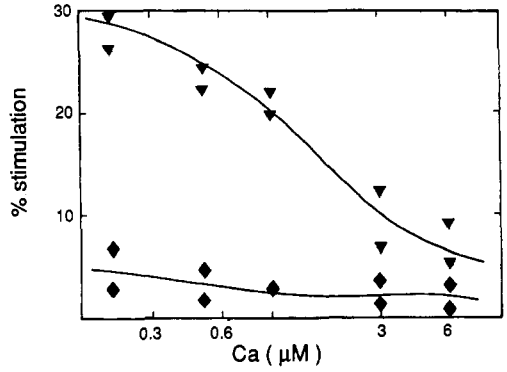


FIGURE 4: $Ca^{2+}$ dependence of the effect of the SR $Ca^{2+}$-ATPase peptide A45 on the $Ca^{2+}$-uptake rate of cardiac and skeletal muscle SR membranes. The $Ca^{2+}$-transport rates by cardiac SR membranes were determined as described in the legend to Figure 3. The stimulatory effect of the synthetic peptide A45 (0.3 mM) was measured at the free $Ca^{2+}$ concentrations indicated on skeletal muscle SR (◆) and cardiac muscle SR (▼). The data points given refer to two independent experiments.

derived from the domain of the cardiac SR ATPase which was previously shown to interact with nonphosphorylated PLN (James et al., 1989). Since the latter inhibits the ATPase activity of cardiac SR, it was considered likely that these synthetic peptides would reverse the inhibition. At submicromolar $Ca^{2+}$, the longer peptide (A45) indeed induced a clear stimulation of the $Ca^{2+}$ uptake rate by cardiac SR membranes (Figure 3): concentrations of the peptide in excess of 100 μM were required for substantial stimulation. The effect of the shorter peptides (A12 and A21) was not significant. None of the peptides affected the $Ca^{2+}$-uptake kinetics of fast skeletal muscle SR preparations (not shown). The stimulatory effect of A45 was influenced by $Ca^{2+}$ (Figure 4): the stimulation was evident at submicromolar $Ca^{2+}$ and became insignificant at higher $Ca^{2+}$ concentrations, which saturate the transport system. Thus, the peptide increased the apparent $Ca^{2+}$ affinity of the pump but did not affect the $V_{max}$ of its transport.

*Effect of Synthetic Phospholamban Peptides on the $Ca^{2+}$ Transport Activity of Cardiac and Skeletal Muscle SR Membranes.* Since the PLN binding domain is present also in the $Ca^{2+}$-ATPase isoform expressed in fast-twitch muscles

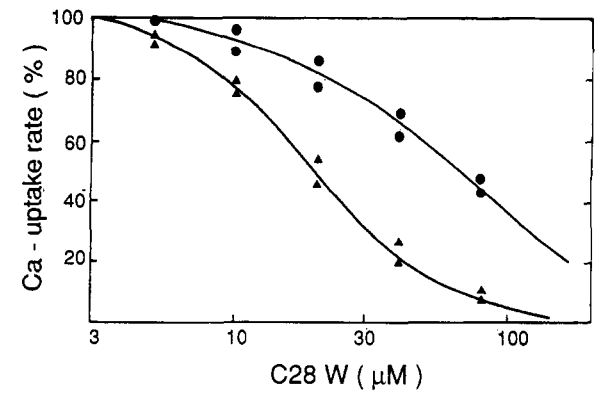


FIGURE 5: Reversal by peptide A45 of the inhibitory effect of peptide C28W on the $Ca^{2+}$ uptake by skeletal muscle SR. Skeletal muscle SR membranes (20 μg/mL) were incubated in the presence of various amounts of peptide C28W without (▲) and with (●) 1.50 μM peptide A45. The $Ca^{2+}$-uptake reaction was started by the addition of ATP as described in the Experimental Procedures section. The free $Ca^{2+}$ concentration was 0.3 μM. The data points given refer to two independent experiments.

(James et al., 1989), PLN should also affect the activity of SR in this type of muscle. The following experiment was carried out to verify this assumption. Three synthetic peptides (P12, P20, and P32), corresponding to various lengths of the hydrophilic domain of cardiac PLN, were used. The longest (P32) represented the entire cytosolic domain extending from the amino terminus to the point of insertion into the membrane. All peptides contained the Ser and Thr residues phosphorylated by the cAMP- and the calmodulin-dependent kinases, respectively. These peptides had a minor effect on the $Ca^{2+}$-uptake rate of skeletal muscle SR. Only very high concentrations of P32 (above 300 μM) produced a small inhibition (less than 20%), which was visible only at low free $Ca^{2+}$ concentrations (not shown). On the other hand, a synthetic peptide derived from the calmodulin binding autoinhibitory domain of the plasma membrane $Ca^{2+}$-ATPase (C28W) proved to be a potent inhibitor; the $IC_{50}$ was about 15 μM, with almost complete inhibition of $Ca^{2+}$ uptake being achieved as submicromolar $Ca^{2+}$ (Figure 5). Recent work has shown that peptide C28W, which is structurally related to PLN, acts on the fast skeletal muscle SR $Ca^{2+}$-ATPase by mimicking the action of PLN (Chiesi et al., 1991). Interestingly, the synthetic peptide derived from the PLN binding domain of the ATPase (A45) reversed the inhibition by peptide C28W. Figure 5 shows that the concentration dependence of the inhibitory action of peptide C28W on the fast skeletal muscle SR $Ca^{2+}$ uptake was shifted to the right in the presence of peptide A45. Although this indicates a competition between peptide A45 and the ATPase for peptide C28W, it could also reflect a decrease of the affinity of the ATPase for peptide C28W due to the binding of peptide A45. However, the data from the CD measurements and the cross-linking experiments support the conclusion that peptides C28W and A45 do indeed interact.

## Discussion

Regulation of the SR $Ca^{2+}$-ATPase by PLN occurs only in the reticulum of slow-twitch and cardiac muscles. However, the ATPase isoform expressed in fast-twitch muscles is highly homologous to the cardiac isoform (Brandl et al., 1987). Although not regulated by the PLN in the SR membrane, the fast-twitch muscle isoform can be specifically cross-linked to photoactivatable PLN (James et al., 1989). Since the regulation of SR $Ca^{2+}$-ATPases apparently depends on the selective expression of PLN in the muscle rather than on intrinsic

differences in the enzymes, it should be possible to regulate the fast-twitch SR ATPase by exogenously added PLN or by synthetic PLN peptides. The present investigation has shown that peptides derived from the hydrophilic portion of PLN inhibited only weakly the fast-twitch skeletal muscle SR pump. A recent study on the reconstituted cardiac muscle SR ATPase has been shown that a similar synthetic peptide (PLN amino acids 1–25) inhibited the $Ca^{2+}$-transport activity (Kim et al., 1990). However, high concentrations of peptide were required for the inhibition (i.e., a peptide to ATPase ratio in excess of 20), and the maximal inhibitory effect was limited to about 20%; obviously the hydrophilic PLN peptides are poor inhibitors for slow- and fast-twitch muscle ATPases. However, native PLN is anchored to the SR membrane via its hydrophobic C-terminal portion; its concentration in the immediate neighborhood of the ATPase would thus be very high and could compensate for the low affinity of its hydrophilic domain. In other words, the inhibitory potency of PLN in situ could be much greater than that expected from the results obtained with the soluble synthetic peptide derivatives. By contrast, the synthetic, calmodulin binding autoinhibitory domain of the plasma membrane $Ca^{2+}$-ATPase, which is structurally related to PLN (Chiesi et al., 1991), had a very potent inhibitory action. Thus, an unspecific binding of this peptide to the $Ca^{2+}$-ATPase is not very likely. The reversal of the ATPase inhibition of peptide C28W upon addition of peptide A45 also indicates that the interaction between these peptides is likely to be specific. Unfortunately, the experimental conditions used to demonstrate the formation of the complex of peptides P32 and A45 in the CD measurements could not be duplicated in the functional ATPase assay system. Thus, the interpretation of the results still has a margin of uncertainty and an unspecific binding of this peptide cannot be ruled out conclusively. The amphipathic $\alpha$-helical character of the autoinhibitory peptide of the plasma membrane ATPase is much more pronounced than that of the hydrophilic portion of PLN. Possibly, this could determine the inhibitory potency and the binding affinity of the peptide.

Previous experiments with a radioactively labeled bifunctional cross-linking agent conjugated to PLN have shown that the latter can be cross-linked to the cardiac SR ATPase, but only in the absence of $Ca^{2+}$ ions and in the nonphosphorylated state (James et al., 1989). The radioactive label was transferred to Lys residues 500 and 503, which are located downstream of the phosphorylation domain and upstream of the ATP binding domain of the ATPase. The region has only negligible homology to other ion-pumping P-type ATPases (Pedersen & Carafoli, 1976) and is partially absent from some. This observation has led to the suggestion that PLN binding could be a peculiarity of sarco(endo)plasmatic reticulum $Ca^{2+}$-ATPases. More recent investigations have shown that the calmodulin binding autoinhibitory domain of the plasma membrane $Ca^{2+}$ pump, which is homologous to the hydrophilic portion of PLN, interacts with the body of the plasma membrane enzyme in a region located between the phosphorylation and the nucleotide binding domains (Falchetto et al., 1991). Therefore, even if the two ATPases have limited sequence homology, they apparently share a structural organization of the regulatory domains which enables them to interact with appropriate amphipathic $\alpha$ helices.

The studies presented here have shown that a 45 amino acid peptide which contains the PLN binding site of the SR $Ca^{2+}$ pump also contains a sequence that can interact with the calmodulin binding autoinhibitory site of the plasma membrane $Ca^{2+}$ pump; the C-terminal portion of peptide A45 became labeled by the photoactivatable calmodulin binding peptide C28W*. It is likely, therefore, that additional residues at the SR pump located N-terminally to the lysines which became labeled with the derivatized PLN (James et al., 1989) are involved in the effects of peptide A45.

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**Registry No.** A45, 137667-94-8; C28W, 120057-55-8; C28W*, 133342-65-1; P12, 137647-98-4; P20, 137647-99-5; P32, 137667-95-9; ATPase, 9000-83-3; Ca, 7440-70-2.

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