Phospholamban Is Related to the Autoinhibitory Domain of the Plasma Membrane Ca^{2+}-Pumping ATPase†

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ABSTRACT: The Ca^{2+} pumps of the plasma membrane (PM ATPase) and of sarcoplasmic reticulum (SR ATPase) share a number of structural and functional properties. A major difference is the regulatory mechanism. The PM ATPase contains a C-terminal autoinhibitory domain; calmodulin binds to it, removing the inhibition. The SR ATPase contains a domain that interacts with the inhibitor protein phospholamban when the latter is in the nonphosphorylated state; phosphorylation of phospholamban removes the inhibition. Peptides corresponding to the autoinhibitory domain of the PM ATPase were synthesized and found to inhibit the SR ATPase. A 28-residue peptide (C28W), containing the entire autoinhibitory domain, was the most powerful (IC_{50} = 15 μM; I_{max} > 90%). The inhibition was Ca^{2+} dependent and more pronounced at submicromolar Ca^{2+} concentrations. C28W is about 50% homologous to the cytosolic domain of phospholamban, the hydrophilic portion of which was found to interact directly with calmodulin (K_d = about 700 nM). However, while calmodulin reversed the inhibition of the SR ATPase by C28W, it failed to reverse that induced by nonphosphorylated phospholamban.

The fine and rapid regulation of Ca^{2+} ions in most cells is accomplished by ATPases. Two major classes of Ca^{2+}-pumping ATPases are known: The one expressed in the plasma membrane (PM) is a protein of about 135 kDa, while the ATPase of the endo(sarco)plasmic reticulum (SR) is a protein of about 110 kDa. Both enzymes have been well characterized [for reviews, see Inesi (1985) and Carafoli (1991)] and shown to have striking similarities. The overall reaction mechanism, which involves the formation and hydrolysis of an acid-stable aspartyl phosphate intermediate, is virtually identical. At the structural level, the similarity between the two proteins is also obvious. Although the homology in primary structure is only of about 30%, the predicted secondary structure is much more similar (Verma et al., 1988). One prominent difference is an extension of about 20 kDa in the C-terminal portion of the PM ATPase that contains the calmodulin-binding autoinhibitory domain (James et al., 1988). Controlled proteolysis removes the domain, which would otherwise block either the Ca^{2+}-binding or the catalytic sites of the pump; the truncated pump displays full enzymatic activity. Calmodulin stimulates the PM ATPase by binding to the autoinhibitory domain, presumably removing it from its site of interaction (Enyedi et al., 1989; Vorherr et al., 1990). A recent investigation with synthetic peptides corresponding to the calmodulin-binding autoinhibitory domain has shown that they inhibit the activity of the proteolytically truncated pump (Enyedi et al., 1989; Vorherr et al., 1990). The SR ATPase, on the other hand, does not contain an autoinhibitory domain, does not interact with calmodulin, and is not stimulated by controlled proteolysis. Its activity is under the control of the expression of the regulatory protein phospholamban (Tada et al., 1975; Wray & Gray, 1977). The latter is a membrane-bound complex of five identical subunits of about 6 kDa each (Fujii et al., 1987), which interacts with the SR ATPase and inhibits its transport activity. Phosphorylation of phospholamban removes the inhibition. In this study, the effect of synthetic calmodulin-binding peptides corresponding to the autoinhibitory domain of the PM ATPase have been investigated on the ATPase of fast twitch muscle SR, which lacks endogenous phospholamban. The results have shown that the peptides mimic the inhibitory action of phospholamban.

Experimental Procedures

Materials. Radioactively labeled [γ-^{32}P]ATP and $^{45}CaCl_2$ were obtained from New England Nuclear. 4-Morpholinepropanesulfonic acid (MOPS),[1] ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and the purified catalytic subunit of the cAMP-dependent protein kinase were purchased from Sigma Chemical Co., St. Louis, Mo. Calmodulin isolated from bovine brain was obtained from Fluka, Buochs, Switzerland. Staurosporin was obtained from the Chemistry Department of CIBA-GEIGY, Basel, Switzerland. All other chemicals were of the best quality commercially available.

Biological Material. SR membranes were obtained from rabbit white muscles and from dog hearts as described by Inesi et al. (1973) and Chamberlain et al. (1983), respectively. Membranes were stored in 300 mM sucrose and 10 mM MOPS, pH 7, at −70 °C and thawed immediately before use. The intermediate microsomal fraction, consisting mainly of elements derived from longitudinal and cisternal elements of the SR, was utilized in this study.

Synthetic Peptides. Peptides C14, C15, C15EE, C20W, and C28W and the analogues C28A and C28Y (see Figure 1), corresponding to a portion or to the full length (C28W)

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[1] Abbreviations: DCC, dicyclohexylcarbodiimide; DVB, divinylbenzene, EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; NMP, *N*-methylpyrrolidone; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PTC, phenylthiocarbamyl; PTH, phenylthiohydantoin; tBu, *tert*-butyl; TFA, trifluoroacetic acid.

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C15	L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I
C15EE	L-E-E-G-Q-I-L-W-F-R-G-L-N-R-I
C14	I-Q-T-Q-I-K-V-V-N-A-F-S-S-S
C20W	L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I-Q-T-Q-I-K
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
C28A	L-R-R-G-Q-I-L-A-F-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-S
C28Y	L-R-R-G-Q-I-L-Y-F-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-S
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

FIGURE 1: Structure of the synthetic peptides used in this study. C28W is the full length calmodulin-binding autoinhibitory domain of the human Ca^{2+} ATPase of the plasma membrane. P32 corresponds to the complete hydrophilic domain of phospholamban. The synthesis procedures are described under Experimental Procedures.

of the calmodulin-binding domain of the Ca^{2+} pump, were synthesized on an Applied Biosystems peptide synthesizer model 431 (Foster City, CA) using the Fmoc/tBU strategy with 1-methyl-2-pyrrolidone for coupling and washing according to the standard protocol for the synthesizer. The synthesis of the hydrophilic portion of phospholamban, peptide P32 (see Figure 1), was also performed by employing the standard cycles of the synthesizer. Details of the synthesis, deprotection, and purification for peptides C14, C15, C20W, and C28W and the A and Y analogues are described elsewhere (Vorherr et al., 1990).

Synthesis of Peptide C15EE. The first amino acid was attached according to the cycle for loading the first amino acid onto the (hydroxymethyl)phenoxy(polystyrene) resin (1.0 mmol/g, 1% DVB). The peptide was synthesized with Pmc protection for the Arg residues. A 10-fold excess of the amino acid derivative was used, and a capping cycle employing acetanhydride after each coupling, according to the instructions of the manufacturer, was performed. Cleavage of the peptide was achieved in a mixture of 1.5 mL of TFA, 400 μL of ethanedithiol, and 80 μL water for 2 h at room temperature. From 92.5 mg of Fmoc-deprotected peptide resin, 46 mg of crude peptide and 16.7 mg of purified peptide were obtained after preparative HPLC with buffers A (0.1% TFA in water) and B (0.05% TFA, 50% 1-propanol in water). According to analytical HPLC performed in buffers A and B, the purity of the peptide was greater than 95%. The amino acid analysis agreed with the expected ratios.

Synthesis of Peptide P32. The first amino acid was attached according to the standard cycle after removal of the Fmoc protecting group of the acid-labile amide anchoring group (Rink, 1987) [4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin; 0.48 mmol/g] attached to polystyrene resin (1% DVB). The peptide was synthesized with Pmc protection for the Arg residues and trityl protection for the Asn and Gln residues, starting with 0.06 mmol of resin and a 10-fold excess of the amino acid derivative. A capping cycle employing acetanhydride after each coupling, according to the instructions of the manufacturer, was performed. Cleavage of the peptide was achieved in a mixture of 2 mL of TFA, 100 μL of ethanedithiol, 100 μL water, and 100 μL of methyl sulfide for 2 h at room temperature. From 134 mg of Fmoc-deprotected peptide resin, 53 mg of crude product were obtained after precipitation and washing with ethyl ether. From 40 mg of crude peptide, 5.2 mg of purified peptide were obtained after preparative HPLC using the buffers A and B. According to analytical HPLC, the purity of the peptide was greater than 95%. The amino acid analysis agreed with the expected ratios.

Binding to Dansyl-Calmodulin. Fluorescence measurements were performed with a SPEX Fluorolog 1680 (Metuchen, NJ) double spectrofluorometer connected to a DM1B coordinator. Quartz cuvettes with a path length of 10 mm and a volume of about 3 mL were used. Dilution effects were <3%, and the temperature of the sample was kept at 26 °C. Dansyl-calmodulin was prepared as described earlier (Vorherr et al., 1990). The dansyl moiety of calmodulin was excited at 340 nm, and the peptides were then added. The resolution of the excitation monochromator was set at 8 nm, and the samples were stirred after the addition of the peptides dissolved in doubly distilled water. Spectra were recorded from 400 to 550 nm. The titration of the fluorescence enhancement was performed by recording the emission at 490 nm. One data point corresponds to fluorescence intensities integrated over a total integration time of 2 s after equilibration of the mixture. For the determination of the affinity constants, the data points were calculated according to Stinson and Holbrook (1973) since the method is more suitable for the determination of boundary values.

Ca^{2+} Uptake. The activity of the Ca^{2+}-pumping ATPase was measured at 37 °C with the Millipore filtration technique and ^{45}Ca (Chiesi & Inesi, 1979). SR membranes, at concentrations ranging from 10 to 50 μg/mL, were incubated in the uptake medium (100 mM KCl, 300 mM sucrose, 5 mM $MgCl_2$, 50 mM MOPS, pH 7.0, 5 mM NaN_3, 3 mM potassium oxalate, 0.5 mM EGTA, and various Ca^{2+} concentrations) to give the Ca^{2+} free values reported in the text which were calculated according to Fabiato and Fabiato (1979). When required the synthetic inhibitory peptides were added to the preincubation buffer. Reversal of the inhibition was studied by adding various amounts of calmodulin together with the inhibitory peptides. After a 10-min preincubation, 2 mM ATP was added to start the uptake reaction. To investigate the effect of phosphorylation on the inhibitory activity of the peptides, the synthetic peptides were added to the preincubation buffer supplemented with 10 mM ATP and various amounts of the catalytic subunit of the cAMP-dependent protein kinase. Ca^{2+} uptake was then measured as described above, but the reaction was started by the addition of SR membranes. The stimulatory effect of calmodulin on cardiac SR was investigated similarly. To eliminate the stimulation of the endogenous calmodulin-dependent protein kinase, 200 nM staurosporin was included in the incubation buffer.

Analysis of Phospholamban Phosphorylation by the Calmodulin-Dependent Kinase. Cardiac SR was incubated at 37 °C in uptake buffer containing 0.34 μM Ca^{2+} free at a concentration of 0.5 mg/mL. When necessary, 3 μM calmodulin and/or 20–500 nM staurosporin wass added. After a 5-min preincubation, the reaction was started by adding 0.5 mM [γ-^{32}P]ATP with a specific activity of 0.2 μCi/nmol. After a 10-min incubation, aliquots were blocked in SDS-containing sample buffer and analyzed by SDS gel electrophoresis. 15% polyacrylamide gels were prepared and run according to Laemmli (1970). After being stained for protein with Coomassie blue and dried, the gels were autoradiographed in the presence of an amplifying screen.

Ca^{2+} ATPase Measurements. The Ca^{2+}-dependent SR ATPase activity was measured at 37 °C in a coupled enzyme assay (Hardwicke & Green, 1974). The reaction medium was composed of 100 mM KCl, 10 mM MOPS pH 7, 2 mM $MgCl_2$, 1 mM EGTA, 0.5 mM Tris-ATP, 0.1 mM NADH, 3 mM phosphoenolpyruvate, and 1 unit/mL each of pyruvate kinase and lactate dehydrogenase. Various concentrations of $CaCl_2$ were added to yield the desired free Ca^{2+} concentration. The reaction was started by adding SR membranes (50 μg/mL). The Ca^{2+} ionophore A23187 was always present in the medium to ensure linear reaction rates and to avoid compli-

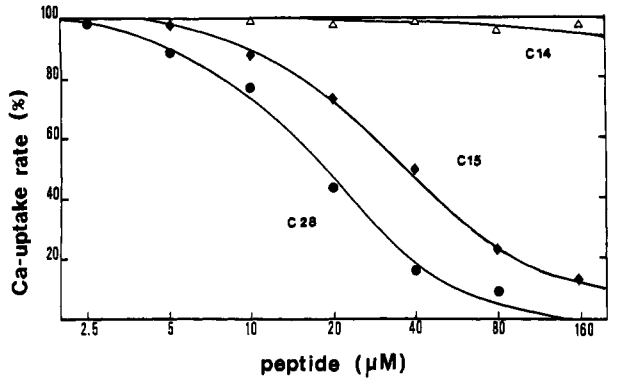

FIGURE 2: Inhibition of SR Ca^{2+} uptake by the autoinhibitory domain of the PM Ca^{2+} ATPase. Ca^{2+} uptake by skeletal muscle SR was carried out at 37 °C by the Millipore filtration technique described under Experimental Procedures. The SR vesicles were preincubated in the presence of the amounts of peptide indicated. The free Ca^{2+} concentration of the uptake medium was 0.3 μM. The rate of Ca^{2+} uptake was determined from the linear phase of the reaction (the first 4 min).

cations due to the modulation of the endogenous Ca^{2+}-release channels by Ca^{2+}.

The Ca^{2+} ATPase activity of the preparation consisting of the 124-kDa proteolytic fragment of the erythrocyte Ca^{2+} pump in the presence and absence of peptides was measured as described by Falchetto et al. (1991). To study the effects of the synthetic peptides on the calmodulin activation of the pump, calmodulin and the peptides were first incubated for 5 min at 37 °C in the assay buffer, and then the enzyme was added and the mixture was incubated another 5 min at 37 °C. The activity was then measured as described above. The free calcium concentration was 10 μM in all experiments. The assay buffer was that described by Falchetto et al. (1991), containing 5 mM $MgCl_2$.

Calpain Digestion of the Ca^{2+} Pump. The purified erythrocyte Ca^{2+} pump was cleaved with calpain to the 124-kDa component as described in Falchetto et al. (1991). Under these conditions of proteolysis, the preparation contains essentially a truncated form of the pump lacking the calmodulin-binding domain and the portion C-terminal to it (James et al., 1989b).

Protein Determination. Protein concentration was determined with the procedure described by Lowry et al. (1951) using bovine serum albumin as a standard.

RESULTS

The SR ATPase of slow twitch muscles like the heart is in a partially inhibited state due to its interaction with endogenous nonphosphorylated phospholamban. Fast twitch skeletal muscle cells do not express phospholamban, and their SR ATPase therefore is in a high-activity state (high affinity for Ca^{2+} and high turnover rate). SR membranes isolated from fast twitch skeletal muscle are thus convenient for investigating the effect of the synthetic peptides corresponding to the autoinhibitory domain of the PM ATPase (see Figure 1 for their sequence). Figure 2 shows that peptide C28W inhibited the Ca^{2+}-uptake reaction of the SR ATPase in a concentration-dependent manner. The IC_{50} was about 16 μM, and the maximal inhibition observed under these conditions (ie., at a free Ca^{2+} concentration of 0.3 μM) exceeded 90%.

The repression of the Ca^{2+}-transport activity by the inhibitory peptide could be due either to the direct inhibition of the turnover rate of the pump or to the uncoupling between its hydrolytic and Ca^{2+}-translocation reactions. Alternatively, it could be due to increased Ca^{2+} permeability of the SR membranes. The effect of the peptides on the rate of the

Table I: Effect of Peptide C28W on the Ca^{2+} ATPase Activity of the SR Pump[a]

	ATPase activity (%)		
	basal rate	low Ca^{2+}	high Ca^{2+}
control	100	100	100
5 μM C28W	95	88	92
10 μM C28W	97	74	88
20 μM C28W	97	55	73

[a] The Ca^{2+} ATPase activity of skeletal muscle SR was measured with a coupled enzyme assay as described under Experimental Procedures. Low Ca^{2+} and high Ca^{2+} corresponded to free Ca^{2+} concentrations of 0.3 and 2 μM, respectively.

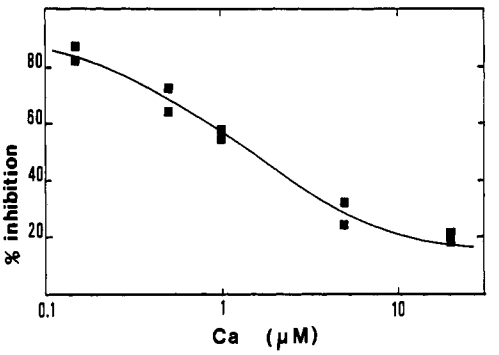

FIGURE 3: Ca^{2+} dependence of the inhibition of Ca^{2+} uptake by C28W. The Ca^{2+}-transport rate of SR membranes from fast skeletal muscle was determined at various free Ca^{2+} concentrations, essentially as described in the legend to Figure 1, in the presence of 40 μM C28W.

Ca^{2+}-dependent ATP hydrolysis catalyzed by the SR ATPase in the absence of a Ca^{2+} gradient across the membrane (i.e., in the presence of the Ca^{2+} ionophore A23187) was thus checked. Under these conditions, the ATPase reaction is not inhibited by negative feedback by the accumulated Ca^{2+} ions, and possible effects of the peptides on the SR Ca^{2+} permeability are thus eliminated. Table I shows that the Ca^{2+} ATPase activity was still inhibited by peptide C28W to a similar extent in the same concentration range, indicating that the peptide directly affected the turnover rate of the enzyme rather than its coupling efficiency or the Ca^{2+}-permeability characteristics of the SR. The inhibition of the Ca^{2+}-dependent ATPase activity was found to be less pronounced at higher Ca^2 concentrations (Table I). The Ca^{2+} dependency of the inhibition was then studied on the Ca^{2+}-transport reaction (Figure 3). At a concentration of 40 μM, the peptide inhibited Ca^{2+} uptake between 20% and 80% depending on the free Ca^{2+} concentration, thus indicating that the peptide affected the V_{max} of the reaction minimally while effectively decreasing the apparent affinity of the ATPase for Ca^{2+}. These inhibitory characteristics recall those of the inhibition of the cardiac SR ATPase by phospholamban (Tada et al., 1978).

Synthetic analogues of C28W in which Trp in position 8 was replaced by Ala or Tyr (peptides C28A and C28Y) have been shown to depress the calmodulin-binding properties of the peptide (Vorherr et al., 1990). They did not significantly alter the inhibitory effects on the SR ATPase (see Table II). The synthetic peptide C15, corresponding to the N-terminal portion of C28W, was also found to inhibit the Ca^{2+}-pumping activity, but its IC_{50} increased slightly to 35 μM (see Figure 1 and Table II). When two positively charged amino acids in peptide C15 (Arg in positions 2 and 3) were replaced by Glu (peptide C15EE), the result was the complete loss of inhibitory activity (see Table II). The C-terminal portion of the peptide C28W (peptide C14) had no significant effect on the transport activity of the SR ATPase up to a concentration of 160 μM.

Table II: Inhibitory Activity of the Synthetic Peptides

	IC_{50} (μM)	
	SR Ca^{2+} pump[a]	PM Ca^{2+} pump[b]
C28W	16	20
C28A	11	40
C28Y	23	5
C15	35	
C15EE	>1000	
C14	>1000	

[a] The Ca^{2+}-uptake rates by skeletal muscle SR membranes were determined as described in the legend to Figure 2 at a free Ca^{2+} concentration of 0.3 μM. The synthetic peptides were added at various concentrations to the preincubation buffer. [b] Proteolytically (calpain) activated PM Ca^{2+} pump. Taken from Falchetto et al. (1991).

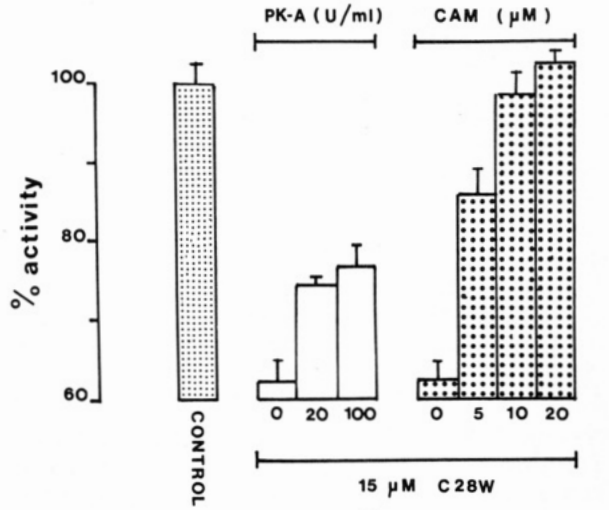

FIGURE 4: Reversal of the inhibition of Ca^{2+} uptake by protein kinase A and calmodulin. The Ca^{2+}-uptake activity of skeletal muscle SR vesicles was investigated at 37 °C in a buffer containing 0.3 μM free Ca^{2+}. When present, C28W was 15 μM. At this concentration, it reduced the uptake activity to about 60% of control. Various concentrations of calmodulin (CAM) or of the catalytic subunit of the cAMP-dependent kinase (PK-A) were induced in the preincubation buffer. When PK-A was used, the peptide was first incubated with it in the presence of ATP, and then the uptake reaction was initiated by the addition of SR membranes. Details are found under Experimental Procedures.

Since C28W contains a possible substrate sequence for the cAMP-dependent protein kinase (R-I-Q-T) the effect of phosphorylation was investigated. Figure 4 shows that preincubation of the peptide with high concentrations of protein kinase A partially reversed the inhibition of Ca^{2+} uptake. No tests were carried out to check whether the peptide had actually become phosphorylated, but the protein kinase did not stimulate the Ca^{2+}-uptake reaction in the absence of the inhibitory peptide.

Complete reversal of the inhibition by C28W was obtained with equimolar concentrations of calmodulin (see Figure 4). Calmodulin has been shown to interact with this synthetic peptide with high affinity (Enyedi et al., 1989; Vorherr et al., 1990) and thus to reverse its inhibition of the PM ATPase. Calmodulin is also known to stimulate Ca^{2+} uptake by cardiac SR by activating the endogenous multifunctional calmodulin-dependent protein kinase, which phosphorylates phospholamban. It would now be tempting to suggest an additional mechanism according to which calmodulin would also stimulate Ca^{2+} uptake by interacting directly with the endogenous inhibitor phospholamban. To test this suggestion, the endogenous calmodulin-dependent kinase was inhibited with 200 nM staurosporin. This concentration had no direct effect on the Ca^{2+}-uptake reaction but completely inhibited the calmodulin-dependent phosphorylation of phospholamban (Figure 5) and the associated stimulation of the uptake (not shown).

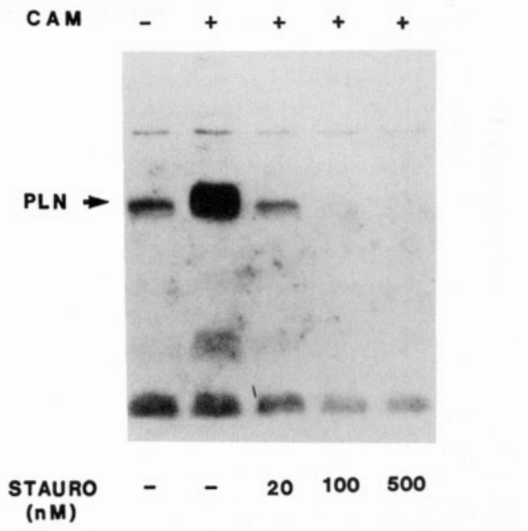

FIGURE 5: Effect of staurosporin on the Ca^{2+} plus calmodulin-dependent phosphoenzyme in cardiac SR membranes. Cardiac SR membranes are phosphorylated with radioactively labeled [γ-^{32}P]ATP in the presence or in the absence of calmodulin and various concentrations of staurosporin as described under Experimental Procedures. The phosphorylation buffer contained 0.45 μM free Ca^{2+}. Phosphorylation was blocked after 2 min at 37 °C, and the phosphorylated proteins were analyzed by autoradiography after separation by SDS–polyacrylamide gel electrophoresis (Laemmli, 1970).

The apparent inability of calmodulin to relieve the inhibition by phospholamban could be due to the insufficient affinity of its interaction with it. Previous cross-linking (Louis & Jarvis, 1982) and gel overlay experiments (Molla et al., 1985) with radioactively labeled calmodulin have suggested a direct interaction between phospholamban and calmodulin that was dependent on the presence of Ca^{2+} ions and was prevented by the phosphorylation of phospholamban. In these studies, however, the affinity of the interaction could not be determined. Direct tests of the interaction of the two proteins with a synthetic 32-residue peptide corresponding to the hydrophilic portion of phospholamban and dansylated calmodulin have confirmed that interaction indeed occurred. Figure 6 shows a plot of the relative fluorescence of dansyl-calmodulin incubated with various concentrations of the synthetic peptide P32. The interaction was Ca^{2+} dependent, and a maximal enhancement factor of about 2.0 was observed in the presence of 0.5 mM Ca^{2+}. The points in the titration curve (see inset) obeyed the equation outlined by Stinson and Holbrook (1973), resulting in a straight line, consistent with a 1:1 binding stoichiometry. A series of experiments under identical conditions produced values of 730 ± 80 nM. The affinity of the interaction was considerably less than that of calmodulin for the autoinhibitory peptide of the PM ATPase, which is in the low nanomolar range (Vorherr et al., 1990).

Peptide P32 was tested for possible inhibitory activity on both the SR and the PM ATPases. Although the skeletal muscle SR ATPase was found to be inhibited by the peptide, the inhibition was very modest, i.e., of the order of 15%, and required high amouts of peptide (>100 μM) (data not shown). A similar weak inhibition had been previously found by Kim et al. (1990), who have investigated the effect of a synthetic peptide corresponding to amino acids 1–25 of PLN on the reconstituted ATPase isolated from cardiac SR.

As expected from the results of Figure 6, which showed that peptide P32 bound to calmodulin, the stimulation of the PM ATPase by calmodulin was decreased somewhat by the pep-

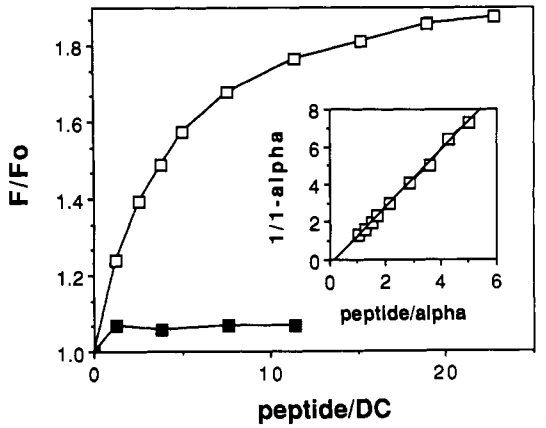

FIGURE 6: Titration of dansyl-calmodulin with peptide P32. Dansyl-calmodulin (190 nM) was titrated with peptide P32 in the presence (□) or absence (■) of Ca^{2+}. Excitation was performed at 340 nm. The relative fluorescence intensities are plotted against the ratio between the total concentration of peptide P32 and the total concentration of dansyl-calmodulin, as given by one representative experiment. The data points were recorded as outlined under Experimental Procedures. (Inset) Calculation of the affinity constant. The fractional degree of saturation of dansyl-calmodulin (alpha) was calculated according to Stinson and Holbrook (1973). The plot of 1/(1-alpha) against the free concentration of peptide (expressed in mM) divided by alpha results in a straight line if a 1:1 complex is formed and the titration end point is correctly estimated. The zero cut on the *X*-axis refers to the total dansyl-calmodulin concentration (190 nM) and serves as a control. The reciprocal of the slope gives the affinity constant. The plot describes one representative experiment. The calculated *K* value was 662 nM.

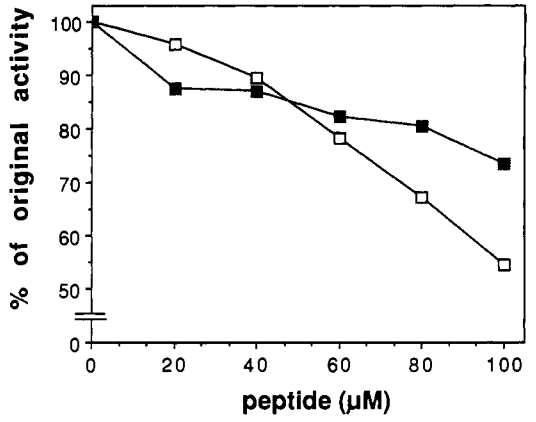

FIGURE 7: Effects of peptides C20W and P32 on the activity of the truncated PM Ca^{2+} pump. The calpain-truncated PM ATPase (15 nM; see Experimental Procedures) was titrated with peptide C20W (□) or peptide P32 (■) in the presence of 10 μM Ca^{2+}. The activity is expressed as percent of the control (no peptide added).

tide. The decrease was very modest and was only evident at high molar ratios (i.e., >20) of peptide to calmodulin.

Recent work in this laboratory (Falchetto et al., 1991) has shown that the calmodulin-binding domain of the PM pump acts as an autoinhibitory sequence. The synthetic peptide C20W, corresponding to the essential portion of the calmodulin-binding domain (Vorherr et al., 1990), indeed inhibited a calpain-proteolyzed preparation of the PM ATPase, which previous work (James et al., 1989b) has shown to have lost the C-terminal portion including the calmodulin-binding domain (Figure 7). Peptide P32 also inhibited the proteolyzed PM ATPase, albeit more modestly.

DISCUSSION

The endogenous regulatory protein phospholamban, which regulates the Ca^{2+} ATPase activity of the SR in slow twitch muscles, is composed of five identical subunits of 52 amino

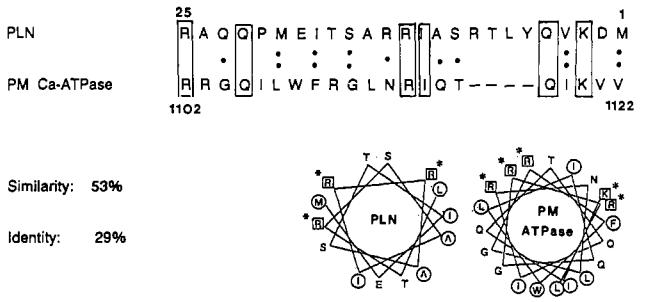

FIGURE 8: Homology between the autoinhibitory domain of the plasma membrane Ca^{2+} ATPase with phospholamban. Alignment of the primary sequence of the autoinhibitory region of the PM Ca^{2+} ATPase (from Arg-1002 to Val-1022) with the sequence of the hydrophilic domain of phospholamban (PLN). The figure also contain a helix-wheel representation of the PM ATPase (from Leu-1101 to Lys-1120) and of phospholamban (from Lys-7 to Met-20, i.e., just before the putative hinge region).

acids (Fujii et al., 1987). The subunits have an amphiphilic configuration (Simmerman et al., 1989): a highly hydrophobic domain at the C-terminus and a 30-residue N-terminal hydrophilic region, probably protruding from the SR membrane. The hydrophilic portion contains the Ser and Thr residues phosphorylated by the cAMP- and calmodulin-dependent kinases, respectively, and is characterized by clusters of positively charged amino acids. This portion of phospholamban is remarkably homologous to the autoinhibitory domain of the PM Ca^{2+} ATPase. Figure 8 shows that the similarity of the primary structure can be extended to the predicted secondary structure. Both polypeptides display a typical amphiphilic α-helical configuration, with most hydrophobic amino acids facing one part of the helix and the positively charged residues facing the other. Since this pattern seems to be a general property of calmodulin-binding domains, it may explain the ability of both polypeptides to interact with calmodulin.

The data presented in this contribution have shown that the synthetic peptides corresponding to the autoinhibitory domain of the PM ATPase are, like phospholamban, very effective inhibitors of the SR ATPase. Their major effect is to decrease the apparent affinity of the ATPase for Ca^{2+}. A reduction in the number of positively charged residues or, possibly, the introduction of negative charges on the synthetic peptide by phosphorylation reverses the inhibition. The data indicate that the mechanism of inhibition is analogous to that by phospholamban. Direct association between phospholamban and the ATPase has been recently demonstrated (James et al., 1989a) and shown to induce a decrease of the apparent affinity of the enzyme for Ca^{2+} (Tada et al., 1979). This could be explained, at least in part, by the introduction of fixed positive charges near the ATPase that would influence the membrane surface potential of the SR and the associated charge-screening effects (Chiesi & Schwaller, 1989; Xu & Kirchberger, 1989). Phosphorylation of phospholamban would counterbalance this effect.

Following the observation by Katz and Remtulla (1978) that calmodulin stimulates the Ca^{2+} translocation into cardiac microsomes enriched in SR membranes, a number of laboratories have investigated this phenomenon and have reached the conclusion that calmodulin acts indirectly, i.e., through the phosphorylation of phospholamban. However, alternative suggestions have also been made. One possibility is that the SR ATPase is stimulated by calmodulin via direct interaction, in analogy to what is known for the PM ATPase. Louis and Jarvis (1982) have shown that cardiac SR contains a 100-kDa protein that can be cross-linked to calmodulin. However, it has been argued (Molla et al., 1985) that the 100-kDa protein

could be contaminating glycogen phosphorylase rather than the ATPase. Alternatively, since phospholamban binds both calmodulin and the SR ATPase, ternary complexes composed of calmodulin–phospholamban–ATPase could be present in the SR membrane, and some ATPase molecules could become cross-linked to calmodulin. In principle, this could also explain the findings of Louis and Jarvis (1982). The results in the present contribution have shown that phospholamban has structural and functional homologies to the autoinhibitory domain of the PM ATPase. Calmodulin reverses the inhibition of the latter by interacting directly with this domain. Thus, it would be conceivable that calmodulin, even though it does not interact with the SR ATPase, could stimulate it by direct interaction with the inhibitor phospholamban, i.e., without phosphorylation of it. However, experiments in which the endogenous calmodulin-dependent kinase was blocked with staurosporin ruled out this possibility, since no effects of calmodulin on the phosphoenzyme (Figure 5) nor on the Ca^{2+}-pumping activity (not shown) were observed under these conditions. Apparently, reversal of the inhibition of the SR ATPase by phospholamban only occurs upon phosphorylation of the inhibitory protein. Thus, even though calmodulin can bind phospholamban in vitro, the interaction might be too weak to occur during the transport cycle of the SR ATPase in situ and thus plays no significant functional role.

The calmodulin-binding domain of the PM Ca^{2+} ATPase acts as an autoinhibitory sequence (see above). Recently, its site of interaction with the main body of the PM ATPase has been identified by using a radioactive derivative of peptide C28W carrying a photoactivatable cross-linking group (Falchetto et al., 1991). The peptide interacted specifically with a region of the pump C-terminal to the aspartic acid that becomes phosphorylated during the reaction cycle. Interestingly, this domain corresponds to the portion of the SR ATPase that was recently shown to interact with nonphosphorylated phospholamban (James et al., 1989a). The finding reported in Figure 7 shows that the hydrophilic portion of PLN apparently interacted with the truncated PM Ca^{2+} pump. The suggestion that the site of interaction was the same as for the autoinhibitory domain of the pump seems very plausible. All these observations support the idea that the regulatory mechanisms of the autoinhibitory domain of the PM ATPase and of phospholamban are similar: The SR ATPase could be visualized as a truncated form of the PM ATPase in which the regulatory protein phospholamban replaces, structurally and functionally, the autoinhibitory domain.

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