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Proteolytic Activation of the Canine Cardiac Sarcoplasmic Reticulum Calcium Pump†

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ABSTRACT: Mild trypsin treatment of canine cardiac microsomes consisting largely of sarcoplasmic reticulum vesicles produced a severalfold activation of oxalate-facilitated calcium uptake. The increase in calcium uptake was associated with an increase in ATP hydrolysis. Proteases other than trypsin were also effective although to a lesser degree. Trypsin produced a shift of the Ca^{2+} concentration dependency curve for calcium uptake toward lower Ca^{2+} concentrations, which was almost identical with that produced by phosphorylation of microsomes by cyclic AMP dependent protein kinase when the trypsin and the protein kinase were present at maximally activating concentrations. The Hill numbers (±SD) of the Ca^{2+} dependency after treatment of microsomes with trypsin (1.5 ± 0.1) or protein kinase (1.7 ± 0.1) were similar and were not significantly different from those for untreated control microsomes (1.6 ± 0.1 and 1.8 ± 0.1, respectively). Autoradiograms of sodium dodecyl sulfate-polyacrylamide electrophoretic gels indicate that ^{32}P incorporation into phospholamban (M_r 27.3K) or its presumed monomeric subunit (M_r 5.5K) was markedly reduced when trypsin-treated microsomes were incubated in the presence of cyclic AMP dependent protein kinase and [γ-^{32}P]ATP compared to control microsomes incubated similarly but pretreated with trypsin inhibitor inactivated trypsin. The activation of calcium uptake by increasing concentrations of trypsin was paralleled by the reduction of phosphorylation of phospholamban. Trypsin treatment of microsomes previously thiophosphorylated in the presence of cyclic AMP dependent protein kinase and [γ-^{35}S]thio-ATP did not result in a loss of ^{35}S label from phospholamban, which suggests that phosphorylation of phospholamban protects against trypsin attack. Trypsin treatment of microsomes prepared from rabbit fast skeletal muscle, which does not contain phospholamban, did not stimulate calcium uptake. However, autoradiograms of gels of rabbit skeletal muscle and canine cardiac microsomes incubated under conditions favorable for the formation of the 100-kilodalton acylphosphoprotein intermediate of the (Ca^{2+} + Mg^{2+})-activated ATPase reaction showed an identical pattern of effects of different concentrations of trypsin. These data suggest that trypsin cleaves similar sites on both calcium pump proteins. Therefore, the stimulatory effect of trypsin on cardiac microsomal calcium uptake does not appear to be due to a direct effect on the calcium pump protein. The data are consistent with a model in which a segment of phospholamban is in communication with the cytoplasm. If the segment is cleaved proteolytically, the basal rate of calcium transport is increased. The proteolytic activation of calcium transport would suggest that the presence of this unphosphorylated cytosolic segment has an inhibitory effect on the calcium pump.

Cardiac muscle relaxation occurs as a result of a decrease in cytoplasmic Ca^{2+} and consequent dissociation of calcium from troponin C. The decrease in cytoplasmic Ca^{2+} is brought about by three different mechanisms. The sodium-calcium exchange system (Reuter & Seitz, 1968) and the sarcolemmal calcium pump (Caroni & Carafoli, 1981) transfer Ca^{2+} out of the cell, and the sarcoplasmic reticulum (SR)[1] calcium pump sequesters calcium within the tubular SR network (Michalak, 1985). The relative contributions of these three

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; SR, sarcoplasmic reticulum; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BAEE, sodium benzoyl-L-arginine ethyl ester; EP, phosphoenzyme intermediate of the sarcoplasmic reticulum (Ca^{2+} + Mg^{2+})-activated ATPase protein; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*'-2-ethanesulfonic acid; TEMED, *N*,*N*,*N*',*N*'-tetramethylethylenediamine; cAMP, adenosine cyclic 3',5'-phosphate; kDa, kilodalton(s).

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systems are difficult to establish. Nevertheless, the role of the SR is an important one because the stored calcium is available for release by the next action potential. The rate of myocardial relaxation is increased in the presence of catecholamines, which stimulate cyclic AMP production and hence activate cyclic AMP dependent protein kinase (Morad & Rolett, 1972).

Previous studies have shown that cyclic AMP dependent protein kinase catalyzed phosphorylation of a low molecular weight protein in SR membranes, named phospholamban, is correlated with an increase in calcium transport by SR membrane vesicles (Kirchberger et al., 1974; Tada et al., 1975) and increased myocardial relaxation in Langendorff heart preparations (Lindemann et al., 1983). Reports from a number of laboratories indicate that phosphorylation of phospholamban may be catalyzed not only by cyclic AMP dependent protein kinase but also by calmodulin- and phospholipid-dependent protein kinases (Le Peuch et al., 1979; Movesian et al., 1984). The roles of the latter two protein kinases in cardiac SR membrane function are poorly understood.

In the present paper, we provide evidence to indicate yet another mechanism by which SR calcium pump activity may be increased. We report severalfold activation of the pump by mild proteolysis and concomitant loss of the ability of phospholamban to be phosphorylated by cyclic AMP dependent protein kinase. Our data support the concept that phospholamban inhibits the calcium pump of the cardiac SR unless phospholamban is in the phosphorylated state and that proteolysis of phospholamban releases the inhibition, thereby circumventing the phosphorylation requirement for stimulation of the pump.

Experimental Procedures

Materials

Acrylamide and *N,N'*-methylenebis(acrylamide) [bis-(acrylamide)] were obtained from Bio-Rad, Richmond, CA. Enzyme-grade urea and sucrose were obtained from Schwarz/Mann Biotech, Cambridge, MA. Cyclic AMP dependent protein kinase was partially purified from bovine heart (Kirchberger et al., 1974). The purified catalytic subunit of the protein kinase was obtained from Sigma Chemical Co., St. Louis, MO. Lyophilized trypsin from bovine pancreas (185 units/mg) and soybean trypsin inhibitor (1 mg inhibits 1.5 mg of trypsin), both obtained from Worthington Biochemical Corp., Malvern, PA, were used in most experiments. In some experiments, bovine pancreatic trypsin supplied by Sigma was used. Xanthine oxidase was obtained from Sigma or Boehringer Mannheim, Indianapolis, IN. Elastase (type IV, 90 units/mg of protein), α-chymotrypsin (type VII, 40–50 units/mg of protein), and papain (type IV, 23 units/mg of protein) were also obtained from Sigma as were Na_2ATP, hydrogen peroxide, and PMSF. PMSF was kept as a 0.28 M stock solution in 2-propanol and was used within 1 h after dilution to 1 mM in the reaction mixture; the vehicle was added as a control. $CaCl_2$ (2 mCi/μmol) was obtained from New England Nuclear. [γ-^{32}P]ATP and [γ-^{35}S]thio-ATP were from Amersham Corp., Arlington Heights, IL. Protein molecular weight standards for gel electrophoresis were insulin (A and B chains) (M_r 3000), bovine trypsin inhibitor (M_r 6000), cytochrome *c* (M_r 12 300), lysozyme (M_r 14 300), β-lactoglobulin (M_r 18 400), α-chymotrypsinogen (M_r 25 700), ovalbumin (M_r 43 000), bovine serum albumin (M_r 68 000), phosphorylase *b* (M_r 97 400), and myosin heavy chain (M_r 200 000), all obtained in kits from Bethesda Research Laboratories, Gaithersburg, MD. The scintillation fluid used was Formula 963 by New England Nuclear, Boston, MA. The filters used in assays of calcium uptake were 0.22-μm pore size GSWP filters obtained from Millipore Corp., Bedford, MA.

Methods

Preparation of Microsomes. Microsomes, consisting largely of fragmented SR, were prepared from canine ventricle as described previously (Kirchberger & Antonetz, 1982a) except that PMSF was omitted from the homogenization and storage buffers. We had previously reported that microsomes prepared in a solution containing 0.3 M sucrose, PMSF, DTT, and buffer were stable over a period of several days when stored on ice (Kirchberger & Antonetz, 1982a). A preliminary experiment was carried out to determine if either PMSF or DTT was necessary for microsomal stability. It was found that calcium uptake activity is lost during the preparation of microsomes in the absence of DTT but that omission of PMSF was without effect. However, PMSF rapidly becomes inactivated in aqueous solutions (James, 1978) and may have been largely inactivated during homogenization of microsomes. DTT was subsequently included in all buffers used for the preparation and storage of microsomes. Microsomes were stored in liquid nitrogen prior to use. They were thawed only once although freezing at least 1 more time had no detectable deleterious effect on calcium transport activity. Protein concentrations were determined by the biuret procedure using bovine serum albumin as the standard.

Assay of Calcium Uptake Activity. Calcium uptake by microsomes was measured at 25 °C in a reaction mixture containing 40 mM histidine–HCl, pH 6.8, 120 mM KCl, 2.5 mM Tris–oxalate, 5 mM NaN_3, 1 mM $MgCl_2$, 1 mM ATP, and a $CaCl_2$–EGTA buffer, prepared as described previously (Kirchberger & Antonetz, 1982a), to give a final $CaCl_2$ concentration of 125 μM and a (free) Ca^{2+} concentration of 0.32 μM in all experiments except one in which a range of Ca^{2+} concentrations extending from 0.02 to 10 μM was utilized. A value of 10^6 M^{-1} was used for the apparent binding constant of Ca^{2+} to EGTA. ^{45}Ca was present at a specific radioactivity of about 1600 cpm/nmol. All reagents were adjusted to pH 6.8 with Tris base or HCl prior to addition to the reaction mixture. Reactions were started by addition of an aliquot of microsomes pretreated with either cyclic AMP dependent protein kinase or protease as described below to give a final microsomal protein concentration of 0.075 mg/mL except where indicated. Aliquots were removed for filtration at 2 and 4 min. Immediately prior to application of the aliquot to the filter, 4 mL of an ice-cold solution of 10 mM Tris-HCl, pH 6.8, and 1 mM $LaCl_3$ was applied to the cup on the filtration manifold so that reactions were stopped by a combination of reduced temperature, dilution of the sample with $LaCl_3$ solution, and filtration, which was complete within 3 s after the aliquot was applied. Filters were then washed with two 2-mL aliquots of the $LaCl_3$ solution, removed from the filtration manifold, and transferred to liquid scintillation vials. One milliliter of ethylene glycol monomethyl ether was added to each vial to dissolve the filter and release the ^{45}Ca from the microsomes. After 30 min, 5 mL of scintillation fluid containing 13 mM $CaCl_2$ was added, and vials were counted in a liquid scintillation spectrometer.

Skeletal muscle microsomes were treated similarly except that the microsomal protein concentration in the incubation mixture was 0.01 mg/mL. Under these conditions, calcium uptake rates of skeletal muscle microsomes were linear with time and protein concentration as was the case with cardiac microsomes at a higher concentration.

Treatment of Microsomes with Proteases. Microsomes were treated with trypsin in a reaction medium consisting of

40 mM histidine–HCl, pH 6.8, 120 mM KCl, and 5 mM NaN_3. The concentrations of microsomes and trypsin are specified in the text. Reactions were carried out at 25 °C for 5 min. They were terminated by addition of trypsin inhibitor added to a final concentration of 0.12 mg/mL. Microsomes were similarly treated with chymotrypsin, papain, and elastase except that chymotrypsin (Sigler et al., 1966) and elastase (Watson et al., 1970) were inhibited by 1 mM PMSF and papain was inhibited by 20 μg/mL hydrogen peroxide (Glazer & Smith, 1965). Inhibitors were present for a 2-min period. Papain (Smith & Parker, 1958), elastase (Kaplan & Dugas, 1969), and chymotrypsin (Schwert & Takenaka, 1955) are essentially maximally active at pH 6.8. Trypsin is considerably active toward lysine substrate at pH 6.8 (the optimum being pH 7.2) but not active toward arginine substrate at pH 6.8 (the optimum being pH 7.2) but not active toward arginine substrate at pH 6.8 (Keilova et al., 1969). Protease protein concentrations were determined from the following extinction coefficients ($cm^{-1}·mg^{-1}·mL$): α-chymotrypsin, A_{280} = 2.075 (Schwert & Kaufman, 1951); papain, A_{278} = 2.5 (Glazer & Smith, 1965); elastase, A_{280} = 1.87 calculated on the basis of data reported by Kaplan and Dugas (1969) and a molecular weight of 25 900 (Gertler & Hofmann, 1967); trypsin, A_{280} = 1.71 (Kunitz, 1947). Assays of trypsin activity using BAEE (Schwert & Kaufman, 1951) or casein (Bergmeyer, 1974) as substrate were carried out as described.

Protein Kinase Catalyzed Phosphorylation of Microsomes. To phosphorylate prior to measurement of calcium uptake, microsomes either treated or untreated with protease and/or protease inhibitor at concentrations specified in the text were incubated for 1 min in the same reaction mixture described for protease treatment except that protease was omitted and 1 mM $MgCl_2$, 1 mM ATP, 25 mM NaF, and either 5 μM cyclic AMP and varying concentrations of bovine cardiac protein kinase (see text) or a control solution consisting of 5 mM histidine–HCl, pH 6.8, were present in addition. Aliquots of the reaction mixture were then added to the calcium uptake reaction mixture to start calcium uptake (see above).

To thiophosphorylate microsomes prior to measurement of calcium uptake, microsomes (1.5 mg/mL) were incubated in the same reaction mixture described above except that 1 mM DTT was present and 1 mM adenosine thiotriphosphate was used instead of ATP. Incubations were carried out in centrifuge tubes in a total volume of 2.0 mL. After 5 min, reactions were stopped by filling the centrifuge tubes with an ice-cold solution containing Hepes–KOH, pH 6.8, 0.25 M sucrose, 5 mM DTT (buffer A), and 25 mM sodium fluoride. After centrifugation at 100000*g* for 35 min at 3 °C, the resultant pellets were suspended in 340 μL of buffer A and utilized for measurement of calcium uptake after the protein concentration was determined.

To phosphorylate microsomes prior to SDS–PAGE, unless otherwise indicated in the text, microsomes (1.5 mg/mL) were incubated at 25 °C in a reaction mixture containing 40 mM histidine–HCl, pH 6.8, 120 mM KCl, 5 mM NaN_3, 25 mM NaF, 0.1 mM EGTA, 1 mM $MgCl_2$, 2 μM cyclic AMP, 1.5 mg/mL cyclic AMP dependent protein kinase or control solution (5 mM histidine hydrochloride, pH 6.8), and either 1 mM [γ-^{32}P]ATP at a specific radioactivity of 4×10^7 cpm/μmol or 1 mM [γ-^{35}S]thio-ATP at a specific radioactivity of 2×10^8 cpm/μmol. Incubation times were 1 min with [γ-^{32}P]ATP and 5 min with [γ-^{35}S]ATP. The volume was 100 μL. Reactions were stopped by addition of 2 mL of a solution containing 10% trichloroacetic acid and 1 mM NaH_2PO_4. Trichloroacetic acid precipitated samples were processed for SDS–PAGE as described previously (Kirchberger & Antonetz, 1982a).

SDS–Polyacrylamide Slab Gel Electrophoresis and Autoradiography. SDS-solubilized microsomes were applied to slab gels 0.15 cm thick, 12 cm high, and 14 cm wide with overlaid 1.5 cm high stacking gels. The separating gels contained 15% (w/v) acrylamide, bis(acrylamide) at a ratio of 37.5:1, 0.375 M Tris-HCl, pH 8.8, 6 M urea, 0.1% SDS, 0.026% TEMED, and 0.026% ammonium persulfate. Separating gels were usually poured 1 day before use. The stacking gel contained 3% acrylamide, bis(acrylamide) in the same ratio as before, 0.125 M Tris-HCl, pH 6.8, 6 M urea, 10% glycerol, 0.1% SDS, 0.05% ammonium persulfate, and 0.4% TEMED. Gels were run at 18 °C at a constant current of 7 mA per gel for approximately 19 h. They were dried on a Hoefer slab gel dryer. Radioactive bands were identified by autoradiography on Kodak X-Omat AR X-ray film with the aid of a Du Pont Cronex Lightning Plus intensifying screen.

Formation of ^{32}P-Labeled EP. To determine the effect of trypsin on EP formation, skeletal or cardiac muscle microsomes (1.5 mg/mL) were subjected to trypsin treatment as described above. After the reaction was stopped with trypsin inhibitor, an aliquot of a reaction mixture favorable for EP formation was added directly to the same tubes containing the trypsin-treated microsomes. This reaction mixture contained the following reagents at the final concentrations indicated: 40 mM histidine–HCl, pH 6.8, 120 mM KCl, 5 mM sodium azide, 1.5 mM $MgCl_2$, 0.17 mM [γ-^{32}P]ATP, and a $CaCl_2$–EGTA buffer resulting in 2.4 μM Ca^{2+}. Reactions, started by addition of ATP, were run at 0 °C for 30 s, which in a preliminary experiment had been found to be the optimal time. Reactions were stopped with 2 mL of ice-cold 10% trichloroacetic acid containing 1 mM ATP and 1 mM NaH_2PO_4, and tubes were centrifuged at 1700*g* for 10 min. Pellets were washed once and then were recentrifuged. Solubilized microsomes were applied to slab gels of the same dimensions as above but containing 5.6% acrylamide at pH 2.4 (Avruch & Fairbanks, 1972). A constant current of 18 mA was applied for about 18 h at 15 °C.

Assay of ATPase Activity. Microsomes (1.5 mg/mL) were incubated in the presence of 0.005 mg/mL trypsin or 0.12 mg/mL trypsin inhibitor (control) as described above. After 5 min, trypsin inhibitor or trypsin was added to experimental and control incubates, respectively. After a further 2 min, an aliquot of the pretreated microsomes was added to a reaction mixture for measuring ATPase activity. This mixture was identical with the one described for measuring calcium uptake except that nonradiolabeled $CaCl_2$ and 1 mM [γ-^{32}P]ATP (6000 cpm/μmol) were substituted for the ^{45}Ca-labeled $CaCl_2$ and the nonradiolabeled ATP, respectively. Samples were removed at 1, 2, 3, and 5 min and processed as described previously (Kirchberger & Antonetz, 1982b) in order to determine rates of ATP hydrolysis. [^{32}P]P_i liberation was determined by a method based on the extraction of a phosphomolybdate complex in isobutyl alcohol. Ca^{2+}-activated ATPase activity is defined as the difference in P_i liberation measured in the presence of 0.32 μM Ca^{2+} and 1 mM EGTA.

RESULTS

Effect of Trypsin or Other Proteases on Calcium Uptake and Ca^{2+}-Activated ATPase Activity. Calcium uptake in cardiac microsomes preincubated with trypsin was stimulated over a wide range of trypsin concentrations (Figure 1). The optimal concentration of trypsin was 0.01 mg/mL, which produced an approximately 3-fold increase in calcium uptake, measured in the presence of 0.32 μM Ca^{2+}. Trypsin con-

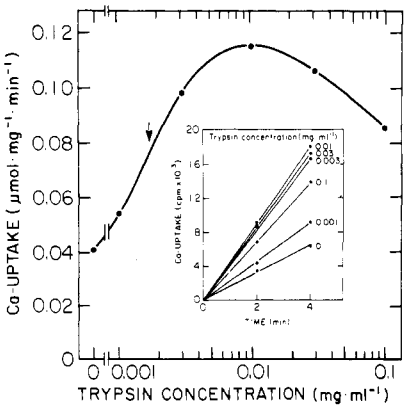

FIGURE 1: Stimulation of calcium uptake by dog cardiac microsomes by trypsin. Microsomes (1.5 mg/mL) were preincubated for 5 min with varying concentrations of trypsin prior to assay of calcium uptake. The arrow indicates the trypsin concentration at which half-maximal stimulation is found (0.0017 mg/mL). The inset shows the raw data from which the calcium uptake rates were derived.

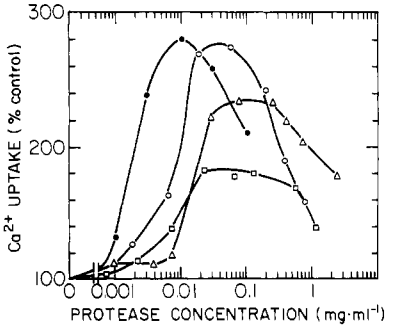

FIGURE 2: Comparison of effects of different proteases on calcium uptake by cardiac microsomes. Microsomes (1.5 mg/mL) were preincubated with trypsin (●), chymotrypsin (○), papain (△), or elastase (□). After the reaction was stopped with the appropriate inhibitor, aliquots were removed for measurement of calcium uptake. Calcium uptake rates in the absence of each protease (controls) were taken as 100%. The absolute calcium uptake rate of the four controls was 0.032 ± 0.002 μmol·mg^{-1}·min^{-1}. Each data point represents the average of two determinations except for trypsin for which data points were taken from Figure 1 for comparison.

centrations above 0.01 mg/mL progressively diminished the extent of stimulation. Concentrations above 0.2 mg/mL trypsin decreased calcium transport below control values (data not shown). The same effect was obtained whether microsomes were present at concentations of 1.5 or 0.088 mg/mL. Trypsin obtained from a different commercial source produced identical results when corrected for differences in specific enzyme activity.

An effect of trypsin on the calcium pump would result in an effect on (Ca^{2+} + Mg^{2+})-activated ATP hydrolysis. In three determinations, (Ca^{2+} + Mg^{2+})-activated ATPase activity (±SD) was 0.06 ± 0.01 μmol·mg^{-1}·min^{-1} in control microsomes (see Methods) and 0.14 ± 0.02 μmol·mg^{-1}·min^{-1} in microsomes treated with 0.005 mg/mL trypsin. Assuming a Ca:ATP ratio of 0.8 (Kirchberger & Antonetz, 1982; Chamberlain et al., 1984), one can calculate rates of calcium uptake of 0.05 and 0.11 μmol of Ca·mg^{-1}·min^{-1} in control and trypsin-treated microsomes, respectively. These figures are similar to those plotted in Figure 1 at 0 and 0.005 mg/mL trypsin.

Various known inhibitors of trypsin activity were then tested. Addition of 0.12 mg/mL trypsin inhibitor or 1 mM PMSF to the reaction mixture had no effect on calcium uptake but prevented the stimulation by trypsin. Since 1 M sucrose has previously been found to prevent or reduce trypsin-induced inhibition of calcium uptake by the SR (Tada et al., 1975; Bidlack & Shamoo, 1980), the effect of sucrose on trypsin activation of calcium transport was tested. One molar sucrose present during preincubation with trypsin shifted the trypsin concentration dependency curve to the right. The inhibitory effect of sucrose was not present when trypsin activity was assayed with BAEE irrespective of whether 120 mM KCl (which was present when microsomes were incubated with trypsin) was included in the assay mixture. However, trypsin activity was inhibited by 1 M sucrose when the protein substrate casein was used.

A number of proteases other than trypsin were tested and also found to produce activation of microsomal calcium uptake although at higher enzyme protein concentrations (Figure 2). These proteases were chymotrypsin, papain, and elastase in decreasing order of effectiveness. In each case, the inhibitor used to stop the protease reaction, namely, PMSF or hydrogen peroxide, had no effect on basal calcium uptake.

Proteolytic contamination of various enzymes used in studies of SR and other biologic systems may result in erroneous interpretations. For example, rather than produce the expected decrease in calcium uptake due to free oxygen radicals (Hess et al., 1981), xanthine oxidase was found to produce a concentration-dependent stimulation as great or slightly greater than that seen with trypsin. The xanthine oxidase was present at 0.01–3.5 units/mL, amounts commonly used in free-radical reactions. This activation of calcium uptake was due to a serine-type protease present in some commercial xanthine oxidase preparations (Ager et al., 1984) since it can be abolished by 1 mM PMSF.

Effect of cAMP-Dependent Protein Kinase on Trypsin Stimulation of Calcium Uptake. To assess the relationship of the stimulation of cardiac microsomal calcium uptake produced by trypsin to that produced by cAMP-dependent protein kinase, microsomes were pretreated with trypsin and then with protein kinase prior to measurement of calcium uptake. In a preliminary experiment, a protein kinase concentration dependency curve was obtained which showed maximum stimulation at 0.6–0.8 mg/mL and approximately half-maximal stimulation at 0.1 mg/mL.

Trypsin present during the first preincubation at a concentration resulting in submaximal stimulation (0.002 mg/mL) and 0.1 and 0.8 mg/mL protein kinase present during the second preincubation each produced the expected enhancement of calcium uptake (Figure 3). However, the stimulation by 0.1 mg/mL protein kinase was essentially eliminated if pretreatment with trypsin took place. Clearly, the stimulatory effects of trypsin and protein kinase were not additive. The maximum stimulation obtained with protein kinase is approximately the same as that obtained with trypsin-pretreated microsomes.

In order to compare further protein kinase stimulated calcium uptake with trypsin-stimulated calcium uptake, we determined the effects of both protein kinase and trypsin, each at maximally stimulating concentrations, on calcium uptake at different Ca^{2+} concentrations (Figure 4). The Ca^{2+} concentration dependency curves obtained with each agent were virtually identical and were shifted to the left by the same amount. Table I shows the apparent $K_{Ca^{2+}}$, V_{max}, and the Hill number, all calculated from the data in Figure 4. Only the apparent $K_{Ca^{2+}}$ changed significantly with protein kinase or trypsin treatment.

Effect of Trypsin on Phospholamban Phosphorylation. Cardiac microsomes, pretreated with and without active trypsin, were incubated under conditions favorable for phospholamban phosphorylation in the presence of [γ-^{32}P]ATP and

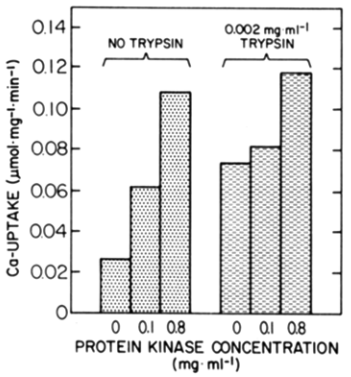

FIGURE 3: Effect of cyclic AMP dependent protein kinase on calcium uptake by cardiac microsomes pretreated with trypsin. Microsomes (1.5 mg/mL) were preincubated for 5 min in either control medium (0.12 mg/mL trypsin inhibitor and 0.002 mg/mL trypsin) or medium containing 0.002 mg/mL trypsin. This preincubation was followed by a 1-min incubation with either distilled water having been added to the controls or 0.12 mg/mL trypsin inhibitor having been added to the microsomes pretreated with trypsin alone. Aliquots were then removed for a further 1-min (phosphorylation) reaction in the presence and absence of 5 μM cyclic AMP and 0.1 or 0.8 mg/mL cyclic AMP dependent protein kinase. The microsomal protein concentration during the latter reaction was 0.68 mg/mL. Finally, aliquots were removed from the phosphorylation or control reaction tubes for assay of calcium uptake.

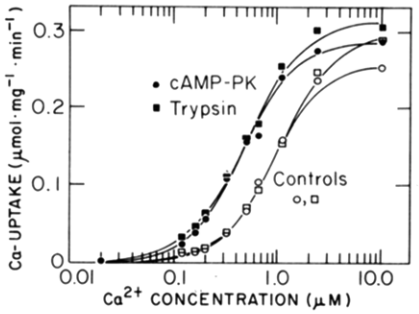

FIGURE 4: Effects of cyclic AMP dependent protein kinase or trypsin on the Ca^{2+} concentration dependency of calcium uptake. Microsomes were pretreated for 5 min in the presence (■) or absence (□) of 0.01 mg/mL trypsin or for 1 min in the presence (●) or absence (○) of 0.8 mg/mL cyclic AMP dependent protein kinase. Aliquots of pretreated microsomes were removed for measurement of calcium uptake. Each data point represents three independent experiments with different microsome preparations.

Table I: Effect of Cyclic AMP Dependent Protein Kinase or Trypsin Treatment on Calcium Uptake by Cardiac Microsomes[a]

	control microsomes	phospho-rylated microsomes	control microsomes	trypsin-treated microsomes
$K_{Ca^{2+}}$ (μM)	0.87 ± 0.04	0.47 ± 0.03[b]	1.05 ± 0.05	0.50 ± 0.03[b]
V_{max} (μmol·mg^{-1}·min^{-1})	0.26 ± 0.01	0.29 ± 0.01[b]	0.30 ± 0.01	0.32 ± 0.01
Hill number	1.8 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.5 ± 0.1

[a] Averages of experimentally measured values for the calcium uptake rates at different Ca^{2+} concentrations obtained in three independent experiments as shown in Figure 4 were fit to the equation $V = V_{max}/[1 + (K_{Ca^{2+}}/[Ca^{2+}])^N]$ by using the nonlinear regression analysis FITFUN on the PROPHET computer system. The Hill number (N) was derived by simultaneously optimizing for $K_{Ca^{2+}}$ and V_{max}; the variance for each data point was used to weight the fit. The standard deviation of data from the fitted curve is presented. [b] $p < 0.05$ when compared to control values using Student's t test for unpaired variates.

the catalytic subunit of cyclic AMP dependent protein kinase. The microsomes were then subjected to SDS–PAGE. Autoradiograms of the gels indicated that trypsin pretreatment of microsomes at 0.01 mg/mL trypsin prevented ^{32}P incorporation into the 27.3- and 5.5-kDa forms of phospholamban compared to microsomes preincubated under similar conditions but in the presence of trypsin inhibitor inactivated trypsin (Figure 5). When the SDS-solubilized microsomes were boiled prior to application to the gel, the radioactivity shifted from the 27.3-kDa region to the 5.5-kDa region on the gel, the presumed monomeric form of phospholamban (Kirchberger & Antonetz, 1982b). The prominent spot of radioactivity in the region on the gel corresponding to a molecular size of 3–5 kDa consists largely of polyphosphoinositides (unpublished observations), which appear also to be reduced in amount following trypsin treatment. The decrease in counts seen in this spot is an additional effect of trypsin on the microsomes, which bears no obvious relationship to phospholamban (unpublished observations). The sensitivity to trypsin of the ability of phospholamban to be ^{32}P labeled (Figure 6) was similar to the trypsin sensitivity of the calcium uptake process (cf. Figure 1).

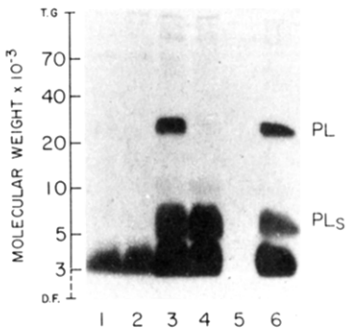

FIGURE 5: Effect of trypsin on ^{32}P labeling of phospholamban observed in an autoradiogram of an SDS–polyacrylamide electrophoretic gel. Cardiac microsomes (1.5 mg/mL) were incubated for 5 min in a reaction mixture containing 40 mM histidine–HCl, pH 6.8, 120 mM KCl, 5 mM NaN_3, and either 0.01 mg/mL trypsin or 0.12 mg/mL trypsin inhibitor plus 0.01 mg/mL trypsin. After 5 min of incubation, trypsin inhibitor was added to those tubes containing trypsin alone, and water (control solution) was added to the rest of the tubes. After an additional 2 min, 45-μL aliquots were removed and added to tubes containing 35 μL of a solution containing the following reagents in final concentrations after addition of aliquots: 13 mM $MgCl_2$, 16 mM EGTA, 24 mM NaF, and 350 units/mL protein kinase catalytic subunit. Phosphorylation reactions were started by addition of 20 μL of 1 mM [γ-^{32}P]ATP at a specific radioactivity of 1×10^8 cpm/μmol, resulting in a total volume of 100 μL. The final microsomal protein concentration was 0.68 mg/mL. Reactions were stopped by addition of 40 μL of a solution containing 6.25% SDS to which were added 10% glycerol and 0.005% bromphenol blue (final concentrations) prior to addition of samples to the gel. Track 1: Microsomes were pretreated with trypsin followed first by addition of trypsin inhibitor and second by additions to allow a phosphorylation reaction. Track 2: Same as for track 1 except that SDS-solubilized microsomes were boiled prior to application to the gel. Track 3: Microsomes were pretreated for 5 min in control solution (trypsin plus trypsin inhibitor), phosphorylated, solubilized in SDS, and applied to the gel. Track 4: Same as for track 3 except that SDS-solubilized microsomes were boiled prior to application to the gel. Track 5: Same as for track 3 except that microsomes were omitted during the incubation but were added to the SDS-solubilized sample. Track 6: Microsomes were subjected only to the phosphorylation reaction. Microsomes incubated under identical conditions except that protein kinase was omitted (not shown) showed an identical pattern of phosphorylation except that ^{32}P incorporation into phospholamban PL) was much reduced. The top of the separating gel (T.G.) and the dye front (D.F.) are indicated in this and subsequent figures of gels. Molecular weight standards (not shown) were used to derive the log scale on the left; the dashed line indicates the range in which the relationship between the log of the molecular weights and the mobility of proteins is nonlinear. PL is found at M_r 27 300 and 5500, corresponding to its presumed monomeric subunit (PL_S). Each track contains 60 μg of microsomal protein.

We next reexamined the known effect of trypsin on phosphorylated cardiac microsomes (Tada et al., 1975; Bidlack &

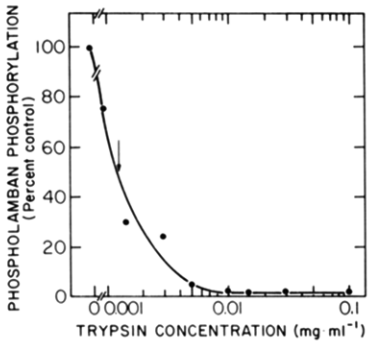

FIGURE 6: Effect of trypsin concentration during pretreatment of microsomes (1.5 mg/mL) with varying concentrations of trypsin on subsequent protein kinase catalyzed ^{32}P incorporation into phospholamban. ^{32}P incorporation into both the 27.3- and 5.5-kDa forms of phospholamban was combined. Microsomes (1.5 mg/mL) were incubated for 5 min in the presence of trypsin, and the reaction were stopped by addition of 0.12 mg/mL trypsin inhibitor. Aliquots were removed and added to reactions mixtures containing buffer solution including 5 μM cyclic AMP, 0.8 mg/mL cyclic AMP dependent protein kinase, and [γ-^{32}P]ATP. The microsomal protein concentration during the phosphorylation reaction was 0.68 mg/mL. Samples were precipitated with trichloroacetic acid and processed for SDS–PAGE. Spots corresponding to phospholamban were cut out and counted. ^{32}P incorporation into microsomes incubated in the presence of trypsin plus trypsin inhibitor prior to phosphorylation was taken as 100% phosphorylation, which corresponds to 0.82 nmol of P_i/mg of microsomal protein. The arrow indicates the concentration of trypsin at which the decrease in ^{32}P incorporation into phospholamban was half-maximal (0.0013 mg/mL).

Table II: Effect of Trypsin on Phosphorylation of Phospholamban[a]

protocol	nmol of P or S·mg^{-1}·mL^{-1}
(1) thiophosphorylation, centrifugation, trypsin, trypsin inhibitor	0.7 ± 0.2
(2) thiophosphorylation, centrifugation, trypsin inhibitor, trypsin	0.7 ± 0.1
(3) centrifugation, thiophosphorylation	0.6 ± 0.1
(4) centrifugation, phosphorylation	1.0 ± 0.2
(5) trypsin, trypsin inhibitor, centrifugation, phosphorylation or thiophosphorylation	nd[b]

[a] Cardiac microsomes were phosphorylated in the presence of cyclic AMP dependent protein kinase and [γ-^{35}S]thio-ATP (thiophosphorylation), centrifuged, and treated with either 0.01 mg/mL trypsin followed by trypsin inhibitor (protocol 1) or trypsin inhibitor followed by trypsin (protocol 2). Additional microsomes were subjected to centrifugation as before and then phosphorylated with cyclic AMP dependent protein kinase and either [γ-^{35}S]thio-ATP (protocol 3) or [γ-^{32}P]ATP (phosphorylation) (protocol 4). Microsomes in protocol 5 were trypsin treated first, centrifuged, and incubated with [γ-^{32}P]ATP and protein kinase. Values are averages of three independent experiments ± SD. [b] Not detectable.

Shamoo, 1980). When ^{32}P-labeled microsomes which had been resedimented by centrifugation and resuspended in buffer were subjected to SDS–PAGE followed by autoradiography, it was found that the amount of ^{32}P label on phospholamban was much reduced, presumably due to phosphoprotein phosphatase activity associated with these membranes (Kirchberger & Raffo, 1977), despite the inclusion of phosphatase inhibitors, namely, fluoride or inorganic phosphate. Therefore, microsomes were thiophosphorylated for 5 min in the presence of [γ-^{35}S]thio-ATP to give the thiophosphate ester, which is highly resistent to phosphatases (Gratecos & Fisher, 1974). Trypsin at 0.01 mg/mL did not release any of the ^{35}S label from phospholamban (Table II). Included in Table II is the amount of phosphorylated phospholamban obtained when [γ-^{32}P]ATP was used as substrate and the microsomes were processed for SDS–PAGE after the phosphorylation reaction was stopped with trichloroacetic acid. The maximum phosphorylation obtained with adenosine thiotriphosphate was consistently lower than that obtained with ATP even when incubations were extended to 10 or 30 min. As an additional control, microsomes were first trypsin treated, followed by treatment with trypsin inhibitor and centrifugation, and then subjected to phosphorylation in the presence of [γ-^{32}P]ATP and cyclic AMP dependent protein kinase. Consistent with the data in Figures 5 and 6, there was no detectable ^{32}P incorporation into phospholamban under these conditions. To establish whether thiophosphorylation of phospholamban was similar to phosphorylation in terms of its effect on calcium uptake, microsomes were incubated for 5 min in the presence and absence of cyclic AMP dependent protein kinase as described above except that nonradioactively labeled adenosine thiotriphosphate was used, and subsequently assayed for calcium uptake. In two experiments, calcium uptake measured at 0.32 μM Ca^{2+} increased from 0.031 and 0.020 μmol^{-1}·mg^{-1}·min^{-1} to 0.088 and 0.076 μmol·mg^{-1}·min^{-1}, respectively, an approximately 3.3-fold increase. These results indicate that thiophosphorylation of phospholamban is qualitatively similar to phosphorylation in stimulating calcium uptake.

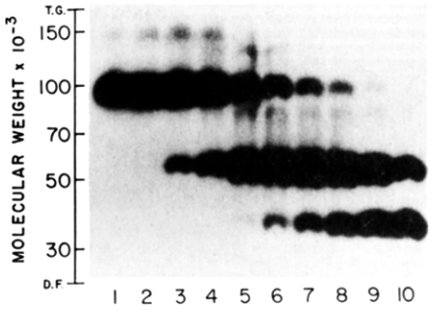

FIGURE 7: Effect of trypsin on subsequent EP formation in cardiac microsomes. Shown is an autoradiogram obtained after SDS–PAGE of microsomes pretreated for 5 min at the following concentrations of trypsin (in milligrams per milliliter): tracks 1 and 2, 0; track 3, 0.001; track 4, 0.002; track 5, 0.004; track 6, 0.01; track 7, 0.02; track 8, 0.045; track 9, 0.1; track 10, 0.3. The above microsomes (1.5 mg/mL) were then incubated in the presence of [γ-^{32}P]ATP under conditions favorable for the formation of the acylphosphoprotein intermediate of the ATPase reaction of the calcium pump protein. Electrophoresis was performed at pH 2.4. Each track contains 150 μg of microsomal protein.

Effect of Trypsin on EP Formation. To examine whether there was a direct effect of trypsin on the 100-kDa calcium pump protein, studies of the effects of trypsin on the subsequent formation of the phosphoenzyme intermediate of Ca^{2+}-ATPase reaction were performed. Cardiac microsomes were pretreated with varying concentrations of trypsin, incubated under conditions favorable for EP formation, and subjected to SDS–PAGE at an acidic pH. Phosphorylation of microsomes in the absence of trypsin pretreatment resulted in essentially all of the ^{32}P label being incorporated into a protein of approximately 100 kDa (Figure 7). In the presence of 0.001 or 0.002 mg/mL trypsin, the lowest concentrations tested, ^{32}P was incorporated into a protein of approximately 55 kDa in addition to the 100-kDa protein. A trypsin concentrations of 0.01 mg/mL and above, there was a progressive increase in the amount of protein incorporated into a 35-kDa protein.

Skeletal muscle microsomes were also pretreated with trypsin and then incubated under conditions favorable for EP formation in the presence of [γ-^{32}P]ATP. Autoradiograms of the dried gel produced an almost identical pattern of ^{32}P incorporation compared to the cardiac microsomes. The effect of different concentrations of trypsin on calcium uptake by skeletal muscle microsomes was determined under conditions

identical with those utilized for cardiac microsomes. In contrast to cardiac microsomes (Figure 1), skeletal muscle microsomes exhibited no stimulation of calcium uptake by trypsin at concentrations of 0.001–0.01 mg/mL. Calcium uptake was inhibited in these microsomes at trypsin concentrations greater than 0.01 mg/mL, which was the same concentration at which the stimulation of calcium uptake in cardiac microsomes by trypsin was decreasing below maximum.

Discussion

Calcium uptake by the cardiac SR is significantly increased upon treatment of SR membranes with trypsin at concentrations ranging over a hundredfold (Figure 1). The effect of trypsin on calcium uptake is clearly an effect on the calcium pump because trypsin produces a proportionate effect on Ca^{2+}-activated ATPase activity. Proteolytic enzymes other than trypsin were also stimulatory (Figure 3). One molar sucrose shifts the trypsin concentration dependency curve to the right. Thus, the degrees of both activation and inhibition at relatively lower and higher trypsin concentrations, respectively, decrease in the presence of 1 M sucrose. These findings are in accord with earlier findings of a protective effect of sucrose against the inhibitory effects of trypsin (Ikemoto et al., 1971; Tada et al., 1975; Bidlack & Shamoo, 1980). The work of Lee and Timasheff (1982) indicates that sucrose increases the activation energy of thermal unfolding of proteins. Therefore, sucrose may affect the conformation of proteins in such a way as to make them less vulnerable to trypsin attack, an effect which would depend on the size and nature of the substrate.

The stimulation of calcium transport by trypsin found in this study and the previously established stimulation by cyclic AMP dependent protein kinase (Tada & Katz, 1982) may be mediated by a common effector. It is seen from the data in Figure 3 that the stimulations by protein kinase and by trypsin are not additive and that trypsin does not further increase maximum stimulation by protein kinase. In addition, the rate of calcium uptake as a function of Ca^{2+} concentration is the same whether trypsin or protein kinase, each at optimal concentration, is used as the stimulatory agent (Figure 4 and Table I).

The effect of protein kinase on calcium transport is known to be mediated by the phosphorylation of phospholamban. Thus, to further investigate the present problem, the effect of trypsin on the phosphorylation of phospholamban was determined by gel electrophoresis. In the presently used gel system, the molecular weight of undissociated phospholamban is 27 300 (Figure 5, tracks 3 and 6), in agreement with that reported by Wegener and Jones (1984), and the molecular weight of the presumed monomeric subunit is 5500, which is similar to the most recent estimate by Jones et al. (1985). Treatment of microsomes with trypsin under conditions where there is a stimulation of calcium uptake results in a loss of the ability of phospholamban to be phosphorylated (Figure 5, tracks 1 and 2 vs. tracks 3 and 4). Although there is also reduced ^{32}P incorporation into polyphosphoinositides, it is not likely to account for the decrease in the apparent K_m for Ca^{2+} (Table I) because the amount of polyphosphoinositides formed which might bind Ca^{2+} is relatively small (unpublished observations). Furthermore, Choquette et al. (1984) observed, in fact, a decrease in K_m for Ca^{2+} with the plasma membrane Ca^{2+}-ATPase of erythrocytes with increasing concentrations of polyphosphoinositides. The trypsin concentration dependency of the loss of the ability of phospholamban to be phosphorylated is identical with that of the stimulation of calcium transport (Figure 6). The inability of phospholamban to be phosphorylated plus the extensively documented functional association of phospholamban with the calcium pump (Tada & Katz, 1982) suggests that the stimulatory effect of trypsin is due to an effect on phospholamban.

Further evidence for the common action of trypsin and protein kinase on the regulatory function of phospholamban on calcium uptake is indicated by the fact that the Ca^{2+} concentration dependency curves of trypsin-treated and phosphorylated microsomes are virtually identical (Figure 4 and Table I). In each case, there is a significant decrease in the apparent $K_{Ca^{2+}}$ and little or no effect on V_{max}, as was reported for calmodulin-stimulated calcium uptake by Davis et al. (1983). Also, there is no change in the Hill number with either treatment. The latter finding is contrary to that of Hicks et al. (1979), who reported a decrease in the Hill number with protein kinase. In recalculating their data by their method, we find that the difference in N between control (unphosphorylated) and phosphorylated microsomes disappears if one eliminates the last data point at 30 μM Ca^{2+}, a value which appears low.

The stimulatory effect of trypsin on cardiac microsomes does not appear to be due to a direct effect of trypsin on the calcium pump protein. A comparison of the autoradiograms of EP of cardiac and skeletal muscle microsomes treated with different concentrations of trypsin indicates an essentially identical pattern of proteolysis, but there is no stimulation of calcium uptake in skeletal muscle microsomes while there is a stimulation in cardiac muscle microsomes. Loss of calcium uptake activity in skeletal muscle microsomes, noted initially at a concentration of 0.01 mg/mL trypsin, was evident only at the second cleavage of EP, that is, at the further dissociation of the 55-kDa phosphate-containing fragment to a 35-kDa phosphate-containing fragment, consistent with the findings of Scott and Shamoo (1982). In cardiac SR, one observes a decreased stimulation by trypsin at the same concentration which produces the second cleavage of EP, namely, starting at 0.01 mg/mL. This may be the basis for the inhibition of calcium uptake in cardiac SR seen at higher trypsin concentrations.

Activation of the SR calcium pump by proteases is yet another example of an enzymic process which may be activated by controlled proteolysis. Enzymes activated in this manner are myosin light chain kinase (Tanaka et al., 1980), phosphorylase *b* kinase (Krebs et al., 1964), phosphodiesterase (Cheung, 1967; Tucker et al., 1981), red blood cell (Ca^{2+} + Mg^{2+})-activated ATPase (Taverna & Hanahan, 1980; Sarkadi et al., 1980), and brain calcineurin (Manalan & Klee, 1983). Each of these enzymes is stimulated by calmodulin, and the mechanism of activation by proteolysis may be similar to that of calcineurin. Calcineurin can be activated by tryptic cleavage of a regulatory domain of the enzyme which normally exerts an inhibitory effect except in the presence of calmodulin (Manalan & Klee, 1983). In analogy to proteolytic activation of calcineurin, trypsin treatment of cardiac SR appears to cleave a region of phospholamban which is inhibitory except when phosphorylated, thus resulting in activation of the calcium pump. Other examples of proteolytic activation of enzymes or biological processes are sodium–calcium exchange in cardiac sarcolemmal vesicles (Philipson & Nishimoto, 1982), protein kinase C (Kishimoto et al., 1983), and type I phosphoprotein phosphatase from bovine heart (Li et al., 1985). In these examples, calmodulin appears not to play a role. Whether there is tryptic cleavage of an inhibitory domain of the enzyme which might be activated by agents other than calmodulin is unclear.

Although the physiologic significance of proteolytic activation of the SR calcium pump, if any, has not been determined, lysosomal proteases liberated during ischemically induced myocardial damage or resultant increased cytoplasmic Ca^{2+} may activate Ca^{2+}-activated proteases called calpains (Yoshimura et al., 1983). One or more of these proteases conceivably may activate the SR calcium pump as well as sodium–calcium exchange and the plasma membrane calcium pump, all of which might act in concert to reduce intracellular Ca^{2+} levels elevated as a result of plasma membrane damage.

Le Peuch et al. (1980) and Wegener et al. (1985) have provided evidence that a small taillike segment of phospholamban projects from the SR membrane into the cytoplasm, which may provide a sensor of the cytoplasmic environment. Le Peuch et al. (1980) reported that this segment contains the phosphorylation sites and is accessible to proteases as demonstrated by a loss of ^{32}P label from phosphorylated phospholamban. This latter result is at variance with earlier studies (Tada et al., 1975; Bidlack & Shamoo, 1980) and the present study in which it was found that only unphosphorylated phospholamban is sensitive to proteolytic attack as determined by its inability to be phosphorylated after trypsin treatment and its inability to be subjected to proteolysis after thiophosphorylation (Table II).

The main difference between the study of Le Peuch et al. (1980) and the present study are that they utilized a trypsin concentration of 0.06 mg/mL for periods ranging from 15 min to 24 h, whereas we utilized a trypsin concentration of 0.01 mg/mL for an incubation period of 5 min. Even at the short incubation time of 5 min, at 0.06 mg/mL trypsin there is extensive inhibition of trypsin stimulation of calcium transport. It should be emphasized that under conditions used in our study, the relationship between proteolysis and calcium transport could be determined. However, these conditions result in rapid loss of ^{32}P label from prior-labeled phospholamban due to intrinsic phosphoprotein phosphatase. Accordingly, we determined the effect of proteolysis on phospholamban which had been subjected to prior phosphorylation with a thiophosphate group, which also results in activation of calcium transport, but which is relatively resistant to hydrolysis by phosphatases. Under these conditions, no action of trypsin on phospholamban could be detected. Hence, we conclude that the action of trypsin on phospholamban, under conditions where the relationship to calcium transport can be determined, can occur only when phospholamban is not phosphorylated under the conditions of this study. The study by Wegener et al. (1985) in which purified phospholamban, i.e., not membrane bound, was used provides evidence that a small segment of phospholamban containing less than 15 amino acids and one or more of the phosphorylation sites may be clipped by the same proteases used in our study.

The considerations presented above are consistent with a model in which the "tail" of unphosphorylated phospholamban, which contains at least one of the phosphorylation sites, is available to the cytoplasm for phosphorylation or for proteolysis. Following phosphorylation, the tail may integrate into the membrane or undergo some other change so that it is no longer accessible to proteolytic enzymes. The proteolytic activation of calcium transport suggests that the presence of the unphosphorylated, presumably cytosolic, segment has an inhibitory effect on the calcium pump of cardiac SR.

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Registry No. Ca, 7440-70-2; trypsin, 9002-07-7; protease, 9001-92-7.

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Structure of Pseudobactin A214, a Siderophore from a Bean-Deleterious *Pseudomonas*†

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ABSTRACT: Bean-deleterious *Pseudomonas* A214 produced the extracellular yellow-green, fluorescent siderophore [microbial iron(III) transport agent] pseudobactin A214 under iron-limiting conditions. Pseudobactin A214 has a molecular formula of $C_{46}H_{64}N_{13}O_{22}$ and a molecular mass of 1151 g/mol. Pseudobactin A214 contained an N-blocked linear octapeptide with the amino acid sequence Ser-Ala-Gly-Ser-Ala-*threo*-β-OH-Asp-L-*allo*-Thr-N^{δ}-OH-Orn with a yellow-green, fluorescent quinoline derivative attached via an amide bond to the amino terminus. A succinamide group was linked to carbon 3 of the quinoline derivative. Sequencing was accomplished by two-dimensional NMR spectroscopy and by Edman degradation of smaller peptides obtained from partial acid hydrolysis. Since pseudobactin A214 was not affected by nonspecific proteolytic enzymes, it might contain D-amino acids. The three bidentate iron(III)-chelating groups consisted of a 1,2-dihydroxy aromatic group in the quinoline chromophore, an α-hydroxy acid group present as β-hydroxyaspartic acid, and a hydroxamate group derived from N^{δ}-acetyl-N^{δ}-hydroxyornithine. The chemical structure of pseudobactin A214 is remarkably similar to those of pseudobactin and pseudobactin 7SR1, the siderophores of plant growth promoting and plant-deleterious *Pseudomonas* B10 and *Pseudomonas* 7SR1, respectively.

Specific root-colonizing members of the *Pseudomonas fluorescens–Pseudomonas putida* group (Schroth & Hancock, 1982) enhance the growth of a variety of crops in part by reducing rhizosphere populations of phytopathogenic fungi (Kloepper et al., 1980a) and deleterious rhizobacteria (Suslow & Schroth, 1982a). These beneficial fluorescent pseudomonads exert their plant growth promoting activity in part by producing under iron-limiting conditions extracellular siderophores [microbial iron(III) transport agents] (Neilands, 1981) that efficiently complex environmental iron, making it less available to certain endemic microorganisms, including phytopathogenic fungi, and thus inhibiting their growth (Kloepper et al., 1980a,b). The structure of pseudobactin, the yellow-green, fluorescent siderophore (Teintze et al., 1981) of plant growth promoting *Pseudomonas* B10, is shown in Figure 1.

Deleterious rhizobacteria, not previously recognized as plant pathogens, significantly decrease the growth of sugar beet, bean, or lettuce seedlings (Suslow & Schroth, 1982a). The

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