Purification and Characterization of a Calcium–Calmodulin-Dependent Phospholamban Kinase from Canine Myocardium†

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ABSTRACT: A Ca^{2+}–calmodulin-dependent protein kinase was purified to apparent homogeneity from the cytosolic fraction of canine myocardium, with phospholamban as substrate. Purification involved sequential chromatography on DEAE-cellulose, calmodulin–agarose, DEAE-Bio-Gel A, and phosphocellulose. This procedure resulted in a 987-fold purification with a 5.4% yield. The purified enzyme migrated as a single band on native polyacrylamide gels, and it exhibited an apparent molecular weight of 550 000 upon gel filtration. Gel electrophoresis under denaturing conditions revealed a single protein band with M_r 55 000. The purified kinase could be autophosphorylated in a Ca^{2+}–calmodulin-dependent manner, and under optimal conditions, 6 mol of P_i was incorporated per mole of 55 000-dalton subunit. The activity of the enzyme was dependent on Ca^{2+}, calmodulin, and ATP·Mg^{2+}. Other ions which could partially substitute for Ca^{2+} in the presence of Mg^{2+} and saturating calmodulin concentrations were $Sr^{2+} > Mn^{2+} > Zn^{2+} > Fe^{2+}$. The substrate specificity of the purified Ca^{2+}–calmodulin-dependent protein kinase for cardiac proteins was determined by using phospholamban, troponin I, sarcoplasmic reticulum membranes, myofibrils, highly enriched sarcolemma, and mitochondria. The protein kinase could only phosphorylate phospholamban and troponin I either in their purified forms or in sarcoplasmic reticulum membranes and myofibrils, respectively. Exogenous proteins which could also be phosphorylated by the purified protein kinase were skeletal muscle glycogen synthase > gizzard myosin light chain > brain myelin basic protein > casein. However, phospholamban appeared to be phosphorylated with a higher rate as well as affinity than glycogen synthase. These findings suggest that a Ca^{2+}–calmodulin-dependent protein kinase, composed of 10 identical subunits, may participate in regulation of sarcoplasmic reticulum function in cardiac muscle.

Biochemical responses to regulatory stimuli are mediated by at least two intracellular signals, cAMP and calcium. Available evidence from in vitro and in vivo studies suggests that the responses to these second messengers may be interrelated. This interrelationship is particularly evident at the level of phospholamban, which is an integral proteolipid of cardiac sarcoplasmic reticulum and is the putative regulator for the Ca^{2+}-ATPase. Phospholamban may be phosphorylated by cAMP-dependent (Kranias et al., 1980; Tada & Katz, 1982; Kranias, 1985), Ca^{2+}–phospholipid-dependent (Movsesian et al., 1984), and Ca^{2+}–calmodulin-dependent protein kinases (LePeuch et al., 1979; Bilezikjian et al., 1981; Kirchberger & Antonetz, 1982; Kranias, 1985; Gasser et al., 1986). These kinases phosphorylate phospholamban at distinct sites, and each phosphorylation is associated with increases in the initial rates of Ca^{2+} transport by cardiac sarcoplasmic reticulum. The sarcoplasmic reticulum associated Ca^{2+}–calmodulin-dependent protein kinase has recently been partially purified, and it appears to consist of subunits with M_r 56 000 (Molla & Demaille, 1986; Jett et al., 1987).

In heart, there are at least three kinds of protein kinases, which are activated in the presence of calmodulin. These include myosin light chain kinase (Walsh et al., 1979; Wolf & Hoffmann, 1980), phosphorylase kinase (Werth et al., 1982), and a multifunctional Ca^{2+}–calmodulin-dependent kinase II (Kloepper & Landt, 1984; Iwasa et al., 1985, 1986). The multifunctional Ca^{2+}–calmodulin-dependent protein kinases have been shown to have broad substrate specificity, and they are present in several tissues such as brain (Fukunaga et al., 1982; Yamauchi & Fujisawa, 1983; Bennett et al., 1983; Goldenring et al., 1983; Kuret & Schulman, 1984), liver (Ahmed et al., 1982; Payne et al., 1983), and skeletal muscle (Woodgett et al., 1983). These enzymes have several features in common. They are oligomeric proteins of high molecular mass (300–700 000 Da), composed of structurally related subunits (50–60 000 Da). They exhibit similar substrate specificities and undergo a Ca^{2+}–calmodulin-dependent autophosphorylation. However, these kinases differ in the exact molecular weights and number of their subunits. Partial purification of two types of multifunctional calmodulin-dependent protein kinases (M_r 300–700 000) from the cytosolic fraction of bovine myocardium has previously been described (Kloepper & Landt, 1984). These protein kinase preparations exhibited phosphorylation of endogenous substrates with M_r 57 000 and 73 000, and they showed high preference for synapsins IB and IA followed by MAP-2 and glycogen synthase, as substrates. Myelin basic protein and casein were rather poor substrates for these calmodulin-dependent protein kinases, and the purified enzymes appeared to also phosphorylate phospholamban (Kloepper & Landt, 1984). Iwasa et al. (1985, 1986) have also reported the purification of a multifunctional calmodulin-dependent protein kinase from canine cardiac cytosol, using chicken gizzard myosin light chain as substrate. The canine kinase had a molecular weight of 550 000, and it exhibited phosphorylation of endogenous proteins of M_r 55 000. Other preferred substrates for this purified protein kinase were myelin basic protein, MAP-2, casein, and glycogen synthase. Addition of the protein kinase to cardiac sarcoplasmic reticulum enhanced the phosphorylation of phospholamban (Iwasa et al., 1985). Thus, although the isolated Ca^{2+}–calmodulin-de-

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pendent protein kinases from bovine and canine muscle appeared to be different, they both were shown to phosphorylate phospholamban in sarcoplasmic reticulum. Since phospholamban is an important regulatory phosphoprotein in cardiac muscle and it has recently been reported to be phosphorylated by a calcium–calmodulin-dependent protein kinase in intact hearts (Wegener et al., 1987), the purpose of our study was to determine whether the apparently different protein kinases isolated from bovine and canine muscle could be detected in the same tissue, when phospholamban was used as a substrate throughout the purification procedure.

We present evidence indicating that a single form of Ca^{2+}–calmodulin-dependent protein kinase activity may be purified to homogeneity from canine cardiac cytosol, using phospholamban as a substrate. This enzyme has a molecular weight of 550 000, and it is composed of identical autophosphorylatable subunits of M_r 55 000. Some characteristics of the purified kinase with respect to its metal ion dependency and substrate specificity are also presented.

Experimental Procedures

Materials

[γ-^{32}P]ATP was purchased from New England Nuclear. DEAE-cellulose (DE-52), phosphocellulose (P11), and 31ET chromatography paper were obtained from Whatman Biochemicals Ltd (U.K.). DEAE-Bio-Gel A, Bio-Gel A-1.5m gel, Affi-Gel 10, and all electrophoretic reagents including low molecular weight standards (10K–100K) were purchased from Bio-Rad. Myelin basic proteins were purchased from Calbiochem. 1-Oleoyl-2-acetyl-*rac*-glycerol (C18:1,[cis]-9; C2:0) was obtained from Sigma, and phosphatidylserine was purchased from Avanti Polar-lipids, Inc. Thyroglobulin, catalase, aldolase, and ferritin were purchased from Pharmacia Fine Chemicals. All other chemicals were reagent grade. Glycogen synthase purified from rabbit skeletal muscle was generously supplied by Dr. Reimann (Medical College of Ohio). Myosin and myosin light chains isolated from cardiac muscle were generously supplied by Dr. J. Di Salvo (University of Cincinnati). Myosin and myosin light chains isolated from turkey gizzard smooth muscle were a gift from Dr. R. S. Adelstein (NIH). Myofilaments and troponin I purified from canine heart were a gift from Dr. R. J. Solaro (University of Cincinnati).

Methods

Assays for Kinase Activity. The Ca^{2+}–calmodulin-dependent phospholamban kinase activity was determined from the amount of [^{32}P]phosphate incorporated into phospholamban. The assay was performed in a total reaction volume of 100 μL which contained 50 mM Tris-HCl, pH 7.5, 0.5 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA),[1] 5 mM $MgCl_2$, 15 mM 2-mercaptoethanol, 10 mM NaF, and 0.2 mM [γ-^{32}P]ATP (100–150 cpm/pmol). The concentrations of $CaCl_2$ and calmodulin, when present in the assay, were 0.6 mM and 2 μM, respectively, unless otherwise specified. Phospholamban (50 μg of TCA-denatured cardiac SR) was used as a substrate during purification of the enzyme. Reactions were initiated by adding the enzyme, incubated at 30 °C for 5 min or the indicated time period, and terminated by either one of the following methods. In method A, reactions (50 μL) were terminated by the addition of 20 μL of an "SDS stop solution" containing 0.125 M Tris (pH 6.8), 9% SDS, 3 mM EDTA, 10% 2-mercaptoethanol, 20% glycerol, and a trace of bromophenol blue. The samples were subjected to SDS–PAGE followed by autoradiography. In method B, an aliquot (70 μL) of reaction mixture was spotted onto squares (2 × 2 cm^2) of Whatman 31-ET chromatography filter paper and immediately immersed into 10% (w/v) TCA. The filter papers were first washed for 20 min in 10% (w/v) TCA and then 3 times for 20 min each in 5% (w/v) TCA. After a final wash in 95% ethyl alcohol, the papers were dried, placed in 6 mL of Budget Solve, and counted in a Beckman Model LS 8100 liquid scintillation counter. We found method B to be convenient for processing large numbers of reaction assays, and, therefore, it was used during the purification procedure of the enzyme. One unit of enzyme activity is the amount of enzyme which can transfer 1 pmol of ^{32}P/min.

Preparation of Proteins. Canine cardiac sarcoplasmic reticulum (SR) vesicles were prepared by a modification of the Harigaya and Schwartz (1968) procedure as previously described by Kranias et al. (1982). The purity and homogeneity of the membrane preparation were checked by electron microscopy after negative staining, by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and by various enzyme markers assayed in the cell homogenate and various fractions of the preparation. Since purified phospholamban is not readily available, canine cardiac SR was used as a substrate of the enzyme during the purification procedure. The endogenous protein kinase activity was irreversibly denatured either by heat treatment or by using 10% TCA, which was then dialyzed out over 48 h at 4 °C against two changes in 50 mM Tris-HCl, pH 7.5 (4000-fold the sample volume). Phospholamban in TCA-denatured cardiac SR was phosphorylated better than in heat-denatured cardiac SR by the Ca^{2+}–calmodulin-dependent protein kinase. Thus, TCA-denatured cardiac SR was used as a substrate for the kinase, in this study. In some experiments, purified phospholamban, isolated as we previously described (Jakab & Kranias, 1988), or a synthetic peptide (detailed synthesis to be described elsewhere) corresponding to amino acids 1–25 of phospholamban was used as substrate. The synthetic peptide was used in kinetic studies, in order to avoid interference by the detergent present in the purified phospholamban preparation. The purified phospholamban or the phospholamban peptide could be phosphorylated to a level of 1 mol of P_i/mol of protein, and this degree of phosphorylation was 7-fold higher than for the phospholamban present in TCA-denatured cardiac SR. Sarcolemmal membranes were isolated from canine cardiac muscle by the method of Van Alstyne et al. (1980) with some slight modifications (Lee et al., 1985). (Na^+, K^+)-ATPase activity was over 100 μmol of P_i mg^{-1} h^{-1}, and [^{3}H]ouabain binding was over 300 pmol/mg. Highly enriched mitochondria were isolated from canine cardiac muscle by the procedure previously described by Matlib et al. (1984). Calmodulin was isolated from freshly collected bovine testes obtained from the Jungman Slaughterhouse, Cincinnati, OH. Briefly, partially purified calmodulin, obtained from the step of DE-52 chromatography using the procedure described by Yazawa et al. (1980), was further purified to homogeneity by sequential chromatography on phenylagarose and Sephadex G-100 (Gopalakrishna & Anderson, 1982).

Preparation of Crude Extract. The ventricular muscle (100 g) of canine heart, freed of fat and fibrous tissue, was minced and homogenized with 400 mL of buffer A (50 mM Tris-HCl,

[1] Abbreviations: SR, sarcoplasmic reticulum; PLB, phospholamban; MLC, myosin light chain; MBP, myelin basic protein; GS, glycogen synthase; SDS, sodium dodecyl sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; TCA, trichloroacetic acid; PMSF, phenylmethanesulfonyl fluoride.

pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 1 mM imidazole hydrochloride) in the presence of protease inhibitors (0.4 mM PMSF, 2 μM pepstatin A, 5 μM antipain, and 15 μM benzamidine) using an Omnimixer at its highest speed for 4 × 15 s. Muscle excision and homogenization were completed within 15 min after removal from the animal, and the speed of completion was critical for maximal recovery of activity. The crude homogenate was centrifuged at 5000*g* for 15 min. The resultant supernatant, referred to as "cell homogenate", was centrifuged at 112000*g* for 45 min. The obtained supernatant is referred to as the "cytosol" and the pellet as the "membrane fraction". The cytosol was filtered through glass wool and used as the starting material for the purification of the Ca^{2+}–calmodulin-dependent protein kinase.

DEAE-cellulose Chromatography. The 112000*g* supernatant or "cytosol" was interacted with 200 mL of DEAE-cellulose, which was preequilibrated with buffer A. After being stirred for 1 h at 4 °C, the gel slurry was poured into a scintered glass funnel. The gel was washed extensively with 20 bed volumes of buffer A containing 50 mM NaCl, and a column (4 × 16 cm) was packed. The bound protein was eluted from the column with a 500-mL linear gradient of 0.05–0.5 M NaCl in buffer A containing 0.4 mM PMSF, 15 μM benzamidine, 5 μM antipain, and 2 μM pepstatin A. Fractions (8 mL) were collected at a flow rate of 60 mL/h. In some experiments, a batchwise elution method was used in order to reduce the time of enzyme purification, which resulted in better recovery of enzymatic activity (see Results). The protein adsorbed on DEAE-cellulose was eluted by combining the washed gel with 500 mL of buffer A containing 0.22 M NaCl, 0.4 mM PMSF, 15 μM benzamidine, 5 μM antipain, and 2 μM pepstatin A. The gel suspension was stirred for 30 min and then filtered through a scintered glass funnel. The filtrate containing enzymatic activity was centrifuged at 112000*g* for 10 min, and the clear supernatant was used for the next step.

Calmodulin Affinity Chromatography. The clear supernatant was adjusted to 3 mM $CaCl_2$ and then applied to a calmodulin–agarose affinity column (2 × 16 cm) equilibrated with buffer B (buffer A containing 3 mM $CaCl_2$). The column was washed with 500 mL of buffer B containing 0.5 M NaCl, followed by 1 column bed volume of buffer B. Protein kinase was eluted with buffer A containing 0.4 mM PMSF and 15 μM benzamidine. Fractions of 5 mL were collected at a flow rate of 30 mL/h.

DEAE-Bio-Gel A Chromatography. Fractions from the calmodulin–agarose column enriched in enzyme activity were pooled and applied to a 1.4 × 15 cm column of DEAE-Bio-Gel A previously equilibrated in buffer A. The column was washed with 20 bed volumes of buffer A, and enzyme activity was eluted with a 100-mL linear gradient of 0–0.4 M NaCl in buffer A containing 0.4 mM PMSF and 15 μM benzamidine. Fractions of 2.5 mL were collected at a flow rate of 10 mL/h.

Phosphocellulose Chromatography. Pooled fractions of the enzyme activity were diluted 2-fold with buffer A before application to a 0.75 × 8 cm column of phosphocellulose previously equilibrated in buffer C (half of the concentration of buffer A). The column was washed with 50 mL of buffer C containing 0.14 M NaCl. Enzyme activity was eluted with 100 mL of buffer C containing 0.2 mM PMSF, 15 μM benzamidine, and 0.4 M NaCl. Fractions of 2.4 mL were collected at a flow rate of 10 mL/h. The fractions enriched in enzyme activity were pooled and concentrated by using an Amicon ultrafiltration unit with a YM-30 membrane. The concentrated protein was dialyzed against 500 mL of 25 mM Tris-HCl, pH 7.5, for 18 h at 4 °C. The dialysate was stored in the presence of 50% glycerol at −20 °C. Under these conditions, the purified kinase could be stored up to 1 month without any appreciable loss of activity.

Molecular Weight Determination. A column (1.5 × 100 cm) of Bio-Gel A-1.5m gel was calibrated by using porcine thyroglobulin (M_r 669000), ferritin (M_r 440000), catalase (M_r 232000), and aldolase (M_r 158000). The void volume (V_0) was estimated by elution of blue dextran 2000. K_{av} values for standards were calculated from their elution volumes (V_e) by the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_t is the total column volume.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of SDS was performed in 0.75-mm-thick slab gels following a modification of the procedure described by Laemmli (1970). The stacking gel contained 3.5% acrylamide [0.09% bis(acrylamide)], and the running gel contained the indicated concentration of acrylamide [0.27% bis(acrylamide)]. Tris–borate buffer (40 mM Tris and 40 mM boric acid, pH 8.64) was used as an electrode buffer instead of Tris–glycine buffer. The gels were stained with Coomassie blue R250, destained, and dried. For autoradiography, the dried gels were exposed to Kodak X-Omat film at room temperature. Autoradiograms were scanned with a Beckman DU-8 spectrophotometer with a white light source. Molecular weight standards were phosphorylase *b* (97400), bovine serum albumin (66200), ovalbumin (42700), carbonic anhydrase (31000), trypsin inhibitor (21500), lysozyme (14400), and aprotinin (6500). Native polyacrylamide tube gel electrophoresis was carried out in 4% acrylamide, by the procedure described above for SDS slab gels but in the absence of SDS.

Miscellaneous Methods. Calmodulin affinity resin was prepared by coupling calmodulin (100 mg) to 50 mL of Affi-Gel 10 (Bio-Rad) resin (90% efficiency) as we previously described (Gupta et al., 1985). Calcium–EGTA buffers contained 1 mM (final concentration) EGTA and various concentrations of $CaCl_2$ as indicated in the text. Free calcium concentrations were calculated by using the association constants of Martell and Smith (1974). A computer program was used to calculate the total concentration of calcium required to obtain the indicated free calcium concentrations at pH 7.5 in the presence of 5 mM Mg^{2+} and 0.2 mM ATP (Martell & Smith, 1974). Calcium concentrations for all solutions were determined by atomic absorption using a Perkin-Elmer 4000 atomic absorption spectrophotometer. Protein determination for the membrane fractions and for the purified protein kinase was obtained by using the Lowry et al. (1951) and the Bradford (1976) methods, respectively.

Results

Identification of Cardiac "Calmodulin-Dependent Phospholamban Kinases". Cardiac sarcoplasmic reticulum contains an integral membrane protein called phospholamban. Cardiac SR may be purified from other subcellular organelles by differential centrifugation. To determine the distribution of "phospholamban Ca^{2+}–calmodulin-dependent protein kinase" activity in canine cardiac muscle, the fractions obtained during the process of SR isolation from muscle homogenates were assayed for Ca^{2+}–calmodulin-dependent protein kinase activity. Since purified phospholamban is not readily available, acid-denatured cardiac SR membranes were used as substrate for the protein kinase activity. The main protein phosphorylated in acid-denatured cardiac SR was phospholamban (M_r 11000). We observed that over 90% of the Ca^{2+}–calmodulin-dependent

Table I: Purification of the Ca^{2+}–Calmodulin-Dependent Protein Kinase from Canine Myocardial Cytosol[a]

step	total protein (mg)	total act. (units)	sp act. (units/mg)	purification (x-fold)	yield (%)
cytosol	2138	44042	20.6	1.0	100
DEAE-cellulose	349	18483	52.96	2.6	42
calmodulin–agarose	1.50	6676	4450	216	15
DEAE-Bio-Gel A	0.46	6066	13130	637	14
phosphocellulose	0.12	2357	20328	987	5.4

[a] Protein kinase activity was assayed at 30 °C in the presence of 0.2 mM [γ-^{32}P]ATP and in the presence or absence of 0.6 mM Ca^{2+} (0.1 mM free) and 1 μM calmodulin. The activity values represent Ca^{2+}–calmodulin-dependent phosphorylation of phospholamban in TCA-denatured cardiac SR. Basal phosphorylation, obtained in the absence of added Ca^{2+} and calmodulin, has been subtracted.

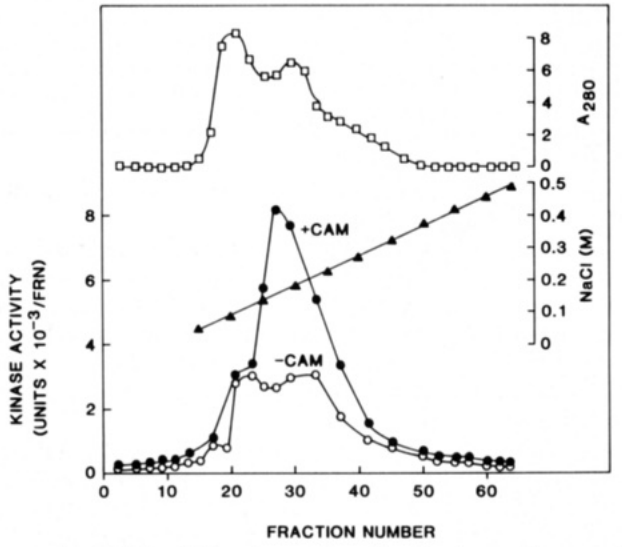

FIGURE 1: DEAE-cellulose chromatography. Canine cardiac cytosol, obtained from 100 g of tissue, was interacted with 200 mL of DEAE-cellulose. The gel was washed, and the bound protein was eluted as described under Experimental Procedures. Fractions were assayed for protein kinase activity in the absence (○) and presence (●) of calmodulin using phospholamban, in TCA-denatured cardiac SR, as substrate (method B). The NaCl concentration (▲) and the absorbance at 280 nm (□) are also indicated.

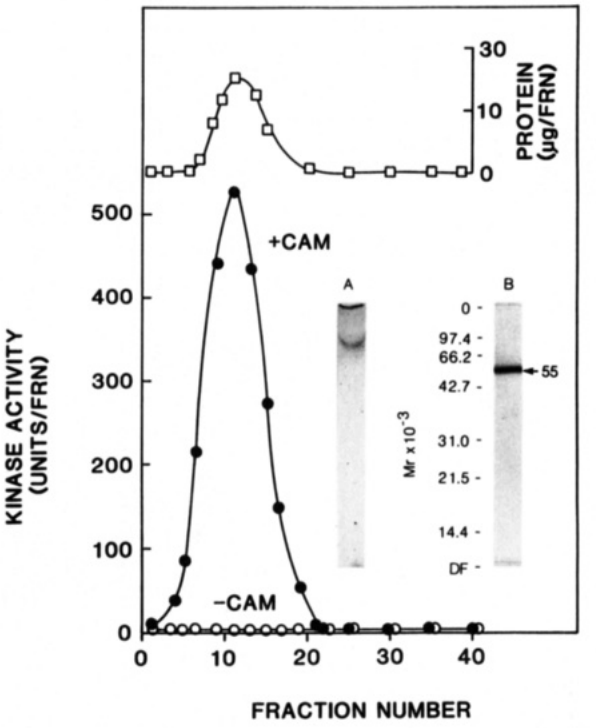

FIGURE 2: Phosphocellulose chromatography. The peak activity fractions from DEAE-Bio-Gel A were diluted 2-fold with buffer A and applied to a column of cellulose phosphate. The column was washed, and the bound protein was eluted as described under Experimental Procedures. Ca^{2+}–calmodulin-dependent kinase activity was determined in the absence (○) and presence (●) of Ca^{2+} and calmodulin as described in Figure 1. Inset: Native (A) and denatured (B) gel electrophoresis of the purified cardiac Ca^{2+}–calmodulin-dependent protein kinase. Purified protein kinase (5 μg) was subjected to electrophoresis on 4% native and 15% SDS gels, as described under Experimental Procedures.

phospholamban kinase present in the cardiac "cell homogenate" was recovered in the "cytosol" and the specific activity of the enzyme in this soluble fraction was comparable to the one present in the pellet, which contained the membrane fraction. Since the cytosol provided a rich source for isolation of the "calmodulin-dependent phospholamban kinase", the enzymatic activity was purified from this fraction of cardiac muscle.

Protein kinases in the cytosolic fraction from canine cardiac muscle were initially characterized by chromatography on DEAE-cellulose. The column was eluted with a NaCl gradient, and fractions were assayed for protein kinase activity in the absence and presence of calmodulin (Figure 1). One major peak of phospholamban Ca^{2+}–calmodulin-dependent protein kinase was observed, and this activity eluted between 0.17 and 0.22 M salt.

Purification of Cardiac "Calmodulin-Dependent Phospholamban Kinase". The enzyme comprising the major peak of calmodulin-dependent phospholamban kinase activity after DEAE-cellulose chromatography was purified further by affinity chromatography on calmodulin–agarose. One major peak of calmodulin-dependent protein kinase activity was observed, and the peak activity fractions were pooled and applied to DEAE-Bio-Gel A. Calmodulin-dependent phospholamban kinase eluted in a symmetrical peak between 0.1 and 0.22 M sodium chloride. The peak activity fractions were pooled, diluted, and applied to a phosphocellulose column (Figure 2). A single symmetrical peak of calmodulin-dependent phospholamban kinase activity was obtained, and the peak activity fractions were pooled, concentrated, and dialyzed before storage at −20 °C in the presence of 50% glycerol.

The purity of the calmodulin-dependent phospholamban kinase was assessed by electrophoresis on 4% polyacrylamide gels under nondenaturing conditions and under denaturing conditions, in the presence of SDS (Figure 2, inset). There was only one protein band observed under each condition, and sodium dodecyl sulfate–PAGE revealed an apparent monomeric molecular weight of 55000. The molecular weight of the holoenzyme was determined by chromatography on a Bio-Gel A-1.5m gel. The enzyme eluted from this column right after the elution of thyroglobulin, and it appeared to have a molecular weight of approximately 550000 (data not shown).

Table I shows the activity of the calmodulin-dependent phospholamban kinase at various stages of the purification procedure. The yield obtained from about 100 g of cardiac cytosol was about 0.12 mg, and the activity appeared to be purified about 1000-fold. Some stimulation of the enzyme by calmodulin was observed in the cytosolic fraction, but a significant and reproducible stimulation was obtained following

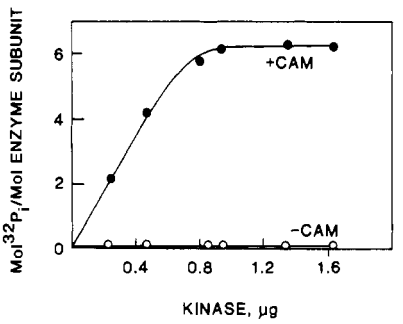

FIGURE 3: Effect of enzyme concentration on autophosphorylation of the purified Ca^{2+}–calmodulin-dependent phospholamban kinase. Different concentrations of the purified kinase were incubated with 0.2 mM [γ-^{32}P]ATP in the absence (○) and presence (●) of Ca^{2+} and calmodulin. Autophosphorylation (10 min at 30 °C) was determined under standard assay conditions using method B, and the stoichiometric incorporation of ^{32}P per enzyme subunit (55 000 daltons) was obtained.

the step of DEAE-cellulose chromatography. The step of calmodulin–agarose chromatography was the most effective in enzyme purification, since most of the protein was not retained on the column at this step. Only those proteins which have an affinity for calmodulin were retained on the column in the presence of Ca^{2+}, and they were eluted with an EGTA buffer. We found that the speed of completion of this step, subsequent to removal of the tissue from the animal, was critical for maximal yields of enzymatic activity. Ca^{2+}–calmodulin-dependent protein kinases are notoriously unstable (Juskevich et al., 1982), and this may be due to various proteases or spontaneous decomposition of the enzyme (Juskevich et al., 1982). Thus, protease inhibitors were routinely included in the homogenization and purification buffers, and the DEAE-cellulose column step was substituted with batch elution of the enzyme with 0.22 M NaCl containing buffer. This allowed completion of the step of calmodulin affinity chromatography within 8 h after tissue homogenization and resulted in maximal yields of enzymatic activity. DEAE-Affi-Gel-Blue chromatography of the calmodulin–agarose-eluted activity failed to further purify the enzyme (data not shown) whereas a 3-fold purification was achieved by DEAE-Bio-Gel A chromatography. At this step, the enzyme was contaminated with myosin light chain kinase, which could be removed by phosphocellulose chromatography (Figure 2). This purification scheme resulted in an enzyme preparation with a 987-fold increase in specific activity and a 5.4% yield (Table I). The purity of the Ca^{2+}–calmodulin-dependent protein kinase was similar to that previously isolated from canine cardiac cytosol (Iwasa et al., 1986), but the yield of the enzymatic activity, employing our purification scheme, was 6-fold higher.

Properties of "Calmodulin-Dependent Phospholamban Kinase". The purified Ca^{2+}–calmodulin-dependent protein kinase activity was independent of several other second messengers. Neither cAMP nor cGMP had a stimulatory effect at concentrations of up to 5 μM. Likewise, Ca^{2+}, 1-oleoyl-2-acetyl-*rac*-glycerol (C18:1,[cis]-9; C2:0), and phosphatidylserine, at concentrations that maximally activate the Ca^{2+}–phospholipid-dependent protein kinase (Movsesian et al., 1984), had no stimulatory effect on the enzyme. These results indicate that the Ca^{2+}–calmodulin-dependent protein kinase is free from other kinase contamination and is composed of approximately 10 identical subunits.

The purified enzyme was autophosphorylated in a calcium–calmodulin-dependent manner, and autophosphorylation was inhibited by trifluoperazine. Under optimal assay conditions, the enzyme incorporated approximately 6 mol of phosphate per mole of its subunits (Figure 3).

Table II: Kinetic Analysis of Selected Ca^{2+}–Calmodulin-Dependent Phospholamban Kinase Substrates[a]

substrate	V_{max} (μmol·min^{-1}·mg^{-1})	K_m (μg/mL)
phospholamban	6.67	10
glycogen synthase	1.428	571
myosin light chain (smooth muscle)	1.29	340.0
myelin basic protein	0.65	140.2
casein	0.13	440.3

[a] Varying concentrations of the different substrates were phosphorylated by 0.1 μg of the purified Ca^{2+}–calmodulin-dependent protein kinase in the presence of Ca^{2+} and calmodulin. The synthetic peptide of phospholamban (see Methods) was used. Incubation was carried out at 30 °C for 1 min, and ^{32}P incorporation into the substrates was determined by method B, as described under Experimental Procedures. Autophosphorylation of the enzyme was subtracted. K_m and V_{max} were calculated by Lineweaver–Burk plots of $1/V$ versus $1/S$.

The amino acid composition of the purified Ca^{2+}–calmodulin-dependent protein kinase was similar to that previously reported for the calmodulin-dependent protein kinase isolated from canine heart using myosin light chain as substrate (Iwasa et al., 1986). The phosphorylatable amino acids, serine and threonine, constitute 6.3% and 4.1% on a molar basis of the holoenzyme.

Substrate Specificity. To determine whether the purified Ca^{2+}–calmodulin-dependent protein kinase was a specific or multifunctional calmodulin-dependent protein kinase type II, the specificity of the enzyme toward several exogenous substrates was assessed. When the same amounts of protein substrate (milligrams per milliliter) were used in the phosphorylation assays, the substrates could be divided in three categories: (1) some proteins (phospholamban, glycogen synthase, gizzard smooth muscle myosin light chain, myelin basic protein, and casein) were phosphorylated with high preference; (2) some (histone III-S, phosvitin, and histone II-AS) demonstrated moderate substrate potential; and (3) some (the histones V-S, VII-S, and II-A, protamine sulfate, phosphorylase *b*, and β-lactoglobulin) did not appear to be phosphorylated by the purified kinase (data not shown). The phosphorylation of each protein was confirmed by autoradiography on SDS–polyacrylamide gels. These results indicate that the purified kinase has broad substrate specificity, but it is distinct from phosphorylase kinase since phosphorylase *b* did not serve as substrate for the purified enzyme.

The substrate specificity of the purified Ca^{2+}–calmodulin-dependent protein kinase was further examined by determining the V_{max} and K_m of the enzyme for the substrates which were phosphorylated with high preference. The data in Table II show that the phospholamban peptide was the most preferred substrate for the purified enzyme (V_{max}, 6.67 μmol of P_i·min^{-1}·mg^{-1}; K_m, 10 μg/mL). The rank order of the other substrates was glycogen synthase, smooth muscle myosin light chain, myelin basic protein, and casein (Table II). To further confirm the specificity of the purified kinase for the various substrates, two sets of experiments were carried out, in which each substrate was phosphorylated by the enzyme and subjected to SDS gel electrophoresis followed by autoradiography. In one set, equimolar concentrations of substrate were used in order to determine the affinity of the enzyme for the various substrates. The phospholamban peptide appeared to be the most preferred substrate for the kinase, followed by glycogen synthase, casein, myelin basic protein, and myosin light chains (data not shown). In another set of experiments, equal amounts of protein were used in the reaction assay in order

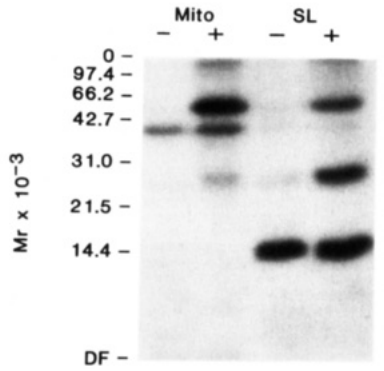

FIGURE 4: Phosphorylation of mitochondria (Mito) and sarcolemmal membranes (SL) by the purified Ca^{2+}–calmodulin-dependent protein kinase. Mito (50 μg) and SL membranes (50 μg) were phosphorylated in the presence of Ca^{2+} and calmodulin and in the presence (+) and absence (–) of 0.12 μg of purified kinase, for 30 s, at 30 °C. The phosphorylated samples were subjected to SDS gel (12%) electrophoresis and autoradiography, as described under Experimental Procedures.

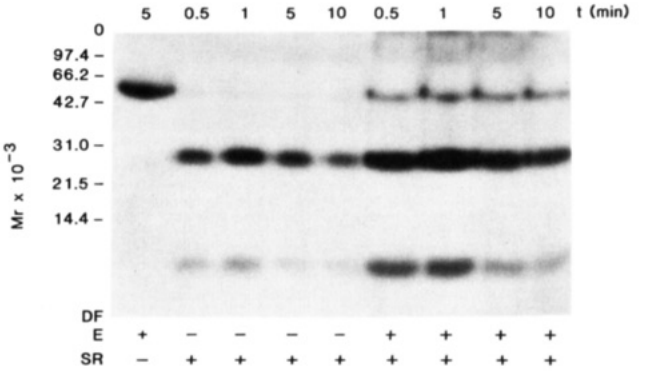

FIGURE 5: Autoradiogram depicting the phosphorylation of canine cardiac sarcoplasmic reticulum by the Ca^{2+}–calmodulin-dependent phospholamban kinase. Sarcoplasmic reticulum (25 μg) was incubated with Ca^{2+} and calmodulin in the absence and presence of 0.12 μg of the purified kinase for the indicated times under standard assay conditions. Forty-microliter aliquots of the samples containing 17 μg of SR were subjected to 10% SDS–PAGE and autoradiography.

to determine the rate by which the enzyme phosphorylates the various substrates (data not shown). The phospholamban peptide appeared to be phosphorylated with the highest rate again, followed by glycogen synthase, myelin basic protein, myosin light chain, and casein. These results are in agreement with the data presented in Table II.

The substrate specificity of the purified Ca^{2+}–calmodulin-dependent protein kinase for endogenous, myocardial proteins was also examined. Troponin I in cardiac myofilaments, which were devoid of endogenous protein kinase activity, could be phosphorylated by the purified kinase. Isolated cardiac troponin I was also phosphorylated by the Ca^{2+}–calmodulin-dependent protein kinase. Phosphorylation was dependent on the concentration of calmodulin in the assay. When the concentration of troponin I was higher than the concentration of calmodulin, it inhibited the enzymatic activity (data not shown). We then examined whether the purified calmodulin-dependent protein kinase could also phosphorylate intact myosin and myosin light chains from the myocardium, since chicken gizzard myosin light chain was one of the preferred substrates of the kinase (Table II). However, there was no observed phosphorylation of cardiac myosin and myosin light chain by the enzyme, indicating that the purified kinase is different from myosin light chain kinase.

The purified Ca^{2+}–calmodulin-dependent protein kinase was also examined for its ability to phosphorylate membrane proteins in isolated mitochondria, sarcolemma, and sarcoplasmic reticulum from cardiac muscle. A protein of M_r 39 000 appeared to be phosphorylated in mitochondria, and inclusion of the calmodulin-dependent kinase did not augment the degree of its phosphorylation (Figure 4). Sarcolemmal membranes appeared to be phosphorylated mainly on a 15 000-Da protein, and addition of the calmodulin kinase was associated with phosphorylation of a 27 000-Da protein, which is probably phospholamban, due to SR contamination (Figure 4). Cardiac SR contains an endogenous Ca^{2+}–calmodulin-dependent protein kinase, which phosphorylates predominantly phospholamban, and addition of the purified enzyme resulted in increased phospholamban phosphorylation (Figure 5). Maximal phosphorylation occurred at 1 min. Further incubation was associated with dephosphorylation of phospholamban (27 000 and 5500 Da) and the autophosphorylated 55 000-Da protein, confirming the presence of a phosphatase which has been reported to be associated with cardiac SR (Kranias & Di Salvo, 1986). The ability of the purified enzyme to phosphorylate a pure phospholamban preparation (Jakab & Kranias, 1988), devoid of endogenous protein kinase activity, was also tested. Autoradiography of SDS gels (Figure 6) showed that the enzyme phosphorylated the 27 000-Da and the 5500-Da species of the purified phospholamban and phosphorylation was dependent on Ca^{2+} and calmodulin. Under optimal assay conditions, there was 1 mol of phosphate incorporated in 1 mol of phospholamban, using a molecular weight of 5500 for the purified phospholamban.

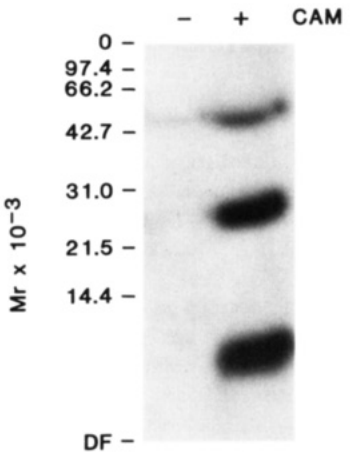

FIGURE 6: Phosphorylation of pure phospholamban by the purified kinase. Pure phospholamban (2.5 μg) was incubated with 0.2 mM [γ-^{32}P]ATP and 0.3 μg of purified kinase in 50-μL assay volume. Phosphorylation was carried out by method A in the absence and presence of Ca^{2+} and calmodulin, under standard assay conditions. An aliquot (35 μL) of unboiled samples containing 1.25 μg of pure phospholamban was subjected to SDS gel (10%) electrophoresis and autoradiography. Pure phospholamban did not contain any kinase activity.

Effect of Cations. Various cations were tested for their effectiveness to substitute for Ca^{2+} in activating the protein kinase. The activity of the purified kinase was assayed in the presence of Mg^{2+}, calmodulin, and 0–10 mM samples of different divalent cations, using phospholamban as substrate (Table III). The apparent EC_{50} values for activation of the enzyme were obtained, and the metals which could substitute for Ca^{2+} were ranked in the order $Sr^{2+} > Mn^{2+} > Zn^{2+} > Fe^{2+}$ (Table III). Hg^{2+} and Cu^{2+} were ineffective.

The effect of various metals on the Ca^{2+}–calmodulin-dependent protein kinase activity was also examined. In these studies, the purified enzyme was preincubated with metals (25 or 500 μM) at 30 °C for 2 min, and reactions were initiated

Table III: Cation Dependence of the Purified Ca^{2+}–Calmodulin-Dependent Protein Kinase[a]

cation added	EC_{50} (μM)	V_{max} (%)
Ca^{2+}	3.2	100
none (Mg^{2+})		1
Sr^{2+}	6.3	92
Mn^{2+}	50	120
Zn^{2+}	251	66
Fe^{2+}	310	43

[a] The purified kinase was assayed as described under Experimental Procedures, using phospholamban in TCA-denatured cardiac SR as substrate, but in the absence of EGTA. Various concentrations (0–10 mM) of different divalent cations were present in the assay. Autophosphorylation of the enzyme was subtracted. Maximal enzymatic activity, obtained in the presence of 0.05 mM $CaCl_2$ (free), was 20.6 nmol of $^{32}P \cdot min^{-1} \cdot mg^{-1}$, and it was taken as 100%.

by adding Ca^{2+}, Mg^{2+}, [γ-^{32}P]ATP, and phospholamban. Preincubation with 25 μM samples of the various cations had no effect on the enzymatic activity. However, preincubation in the presence of higher concentrations (500 μM) was associated with slight stimulation in the case of Sr^{2+} and with inhibition by Fe^{2+} (64%), La^{3+} (52%), and Co^{2+} (37%) (data not shown).

Discussion

Emphasis has recently been placed on isolation and characterization of the Ca^{2+}–calmodulin-dependent protein kinase(s), which show(s) specificity for proteins involved in the regulation of cardiac contractility. This study presents the purification and partial characterization of a multifunctional Ca^{2+}–calmodulin-dependent protein kinase from canine cardiac cytosol, which can phosphorylate phospholamban, the putative regulator for the calcium pump in cardiac SR. We observed that the major portion of the phospholamban Ca^{2+}–calmodulin-dependent protein kinase was cytosolic, and this finding is in contrast to previous ones with other tissues, in which the activity of Ca^{2+}–calmodulin-dependent protein kinases was shown to be predominantly particulate (Landt et al., 1982). The enzyme was identified by its ability to phosphorylate phospholamban, and phospholamban was used as substrate throughout the purification procedure. All chromatographic steps led to elution of a single peak of enzymatic activity, indicating that canine cardiac muscle contains only one major type of Ca^{2+}–calmodulin-dependent protein kinase, which can phosphorylate phospholamban. This observation is similar to that by Iwasa et al. (1986) and different than that by Kloepper and Landt (1984), who identified two Ca^{2+}–calmodulin-dependent protein kinase activities in bovine heart that could phosphorylate phospholamban. The Ca^{2+}–calmodulin-dependent protein kinase activity in this study was purified to homogeneity, and it appeared to consist of identical autophosphorylated subunits of M_r 55 000. This molecular weight of the subunits is similar to that of the Ca^{2+}–calmodulin-dependent protein kinase isolated from canine heart (Iwasa et al., 1986) and one of the two (M_r 57 000 and 73 000) kinase activities isolated from bovine heart (Kloepper & Landt, 1984). The molecular weight of the holoenzyme and its amino acid composition were also similar to those of the calmodulin-dependent protein kinase previously purified from canine heart using myosin light chain as substrate (Iwasa et al., 1986). However, there appeared to be some distinct differences in the properties of the two kinases. These two enzymes had different substrate specificities and differences in the degree of calmodulin dependency for their endogenous phosphorylation. Among four substrates, which were commonly tested, the Ca^{2+}–calmodulin-dependent phospholamban kinase, purified in this study, phosphorylated glycogen synthase with the highest preference followed by smooth muscle MLC, myelin basic protein, and casein, whereas the Ca^{2+}–calmodulin-dependent light chain kinase, previously purified (Iwasa et al., 1986), phosphorylated myelin basic protein with the highest preference followed by smooth muscle MLC, casein, and glycogen synthase. Furthermore, autophosphorylation of the Ca^{2+}–calmodulin-dependent phospholamban kinase, isolated in this study, was totally calcium–calmodulin dependent (0.18 and 11.20 nmol of $^{32}P \cdot mg^{-1} \cdot min^{-1}$ in the absence and presence of Ca^{2+} and calmodulin, respectively), while the autophosphorylation of the Ca^{2+}–calmodulin-dependent light chain kinase, previously isolated (Iwasa et al., 1986), was only slightly stimulated by calcium and calmodulin (31.0 and 34.0 nmol of $^{32}P \cdot mg^{-1} \cdot min^{-1}$ in the absence and presence of Ca^{2+} and calmodulin, respectively).

In cardiac muscle, knowledge of the calmodulin-dependent protein kinases, involved in phosphorylation of regulatory proteins, has been rather limited. Previous studies, on purified calmodulin-dependent protein kinases, have suggested an apparent lack of substrate specificity in vitro, making it difficult to understand the potential physiological significance of these enzymes. Thus, studies as the present one, which utilize cardiac muscle for isolation of the protein kinase and include assessment of substrate specificity using cardiac phosphoproteins, are very valuable. In this context, the calmodulin-dependent protein kinase activity, which we studied, was purified using phospholamban as substrate. We also determined whether several other cardiac proteins may serve as substrates for the purified enzyme. Our findings indicate that the purified Ca^{2+}–calmodulin-dependent protein kinase could phosphorylate troponin I in cardiac myofilaments while there were no observed substrates for this enzyme in isolated sarcolemma and mitochondria from canine cardiac muscle. Cardiac SR and cardiac tropomyosin were previously shown to contain endogenous Ca^{2+}–calmodulin-dependent protein kinase activity (Molla & Demaille, 1986; Iwasa et al., 1986), and it is possible that the soluble enzyme, purified in this study, is an isozyme of the SR-associated and contractile protein associated enzymes. Actually, the SR-associated Ca^{2+}–calmodulin-dependent protein kinase was partially purified, and it was shown to consist of subunits with M_r 56 000. However, the substrate specificity of the membrane-associated enzyme was different (smooth muscle myosin light chain > glycogen synthase > phosphorylase *b* > casein > phosvitin) than the one of the soluble enzyme, which was purified in this study (glycogen synthase > smooth muscle myosin light chain > myelin basic protein > casein).

In view of its physicochemical properties, this kinase belongs to the same class of enzymes recently found to be present in brain (Fukunaga et al., 1982; Yamauchi & Fujisawa, 1983; Goldenring et al., 1983; Bennett et al., 1983; Kuret & Schulman, 1984), liver (Ahmed et al., 1982; Payne et al., 1983), and skeletal muscle (Woodgett et al., 1983), and designated as multifunctional Ca^{2+}–calmodulin-dependent protein kinase type II. They are all high molecular weight enzymes composed of autophosphorylatable subunits with M_r 50 000–60 000 (Goldenring et al., 1983; Woodgett et al., 1983; Kennedy et al., 1983). Furthermore, it has been shown that polyclonal and monoclonal antibodies, raised against the brain Ca^{2+}–calmodulin-dependent protein kinase, cross-react with the myocardial cytosolic Ca^{2+}–calmodulin-dependent protein kinase (Iwasa et al., 1986).

In summary, our findings indicate that a multisubunit Ca^{2+}–calmodulin-dependent protein kinase, which preferen-

tially phosphorylates phospholamban, is present in canine myocardial cytosol. To our knowledge, this is the first report on phosphorylation of pure phospholamban from cardiac muscle by a soluble kinase purified from the same tissue. However, it remains to be seen whether this Ca^{2+}–calmodulin-dependent protein kinase phosphorylates phospholamban in vivo and whether phosphorylation is associated with alterations in sarcoplasmic reticulum function, which may lead to changes in cardiac contractility.

Registry No. GS, 9014-56-6; phospholamban kinase, 85638-42-2.

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