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Characterization and Partial Purification of Cardiac Sarcoplasmic Reticulum Phospholamban Kinase†

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ABSTRACT: Phospholamban, the cardiac sarcoplasmic reticulum proteolipid, is phosphorylated by cAMP-dependent protein kinase, by Ca^{2+}/phospholipid-dependent protein kinase, and by an endogenous Ca^{2+}/calmodulin-dependent protein kinase, the identity of which remains to be defined. The aim of this study was therefore to characterize the latter kinase, called phospholamban kinase. Phospholamban kinase was purified approximately 42-fold with a yield of 11%. The purified fraction exhibits a specific activity of 6.5 nmol of phosphate incorporated into exogenous phospholamban per minute per milligram of protein. Phospholamban kinase appears to be a high molecular weight enzyme and presents a broad substrate specificity, synapsin-1, glycogen synthase, and smooth muscle myosin regulatory light chain being the best substrates. Phospholamban kinase phosphorylates synapsin-1 on a M_r 30 000 peptide. The enzyme exhibits an optimum pH of 8.6, a K_m for ATP of 9 μM, and a requirement for Mg^{2+} ions. These data suggest that phospholamban kinase might be an isoenzyme of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase. Consequently we have searched for M_r 50 000–60 000 phosphorylatable subunits among cardiac sarcoplasmic reticulum proteins. A M_r 56 000 protein was found to be phosphorylated in the presence of Ca^{2+}/calmodulin. Such phosphorylation alters the electrophoretic migration velocity of the protein. In addition, this protein that binds calmodulin was always found to be present in fractions containing phospholamban kinase activity. This M_r 56 000 protein is therefore a good candidate for being a subunit of phospholamban kinase. However, the M_r 56 000 calmodulin-binding protein and the M_r 53 000 intrinsic glycoprotein which binds ATP are two distinct entities.

Muscle relaxation occurs when Ca^{2+} is removed from myofibrils by active transport across the sarcoplasmic reticulum (SR)[1] membrane through a membrane bound (Ca^{2+},Mg^{2+})-ATPase. In cardiac muscle the rate of relaxation is dependent on both the adrenergic status (Wray et al., 1973; Kirchberger et al., 1974) and the cytosolic free Ca^{2+} concentration (Lopaschuk et al., 1980; Plank et al., 1983). These stimulations of Ca^{2+}-dependent ATPase and Ca^{2+} uptake are associated with phosphorylations of phospholamban, a membrane-bound M_r 24 000 proteolipid.

The involvement of these multiple pathways in vivo is under investigation. Although the action of β-adrenergic agonists in systole abbreviation is widely accepted to proceed through phosphorylation of phospholamban (Le Peuch et al., 1980; Kranias & Solaro, 1982), there is no consensus of opinion concerning the importance of phospholamban Ca^{2+}/calmodulin-dependent phosphorylation. Le Peuch et al. (1980) have shown that fluphenazine induces an inhibition of phospholamban phosphorylation in intact rat myocardium. Lindemann and Watanabe (1985) have reported, however, that direct physiological increases in cytosolic free Ca^{2+} do not stimulate phospholamban phosphorylation in vivo. These authors suggest that the Ca^{2+}/calmodulin-dependent phosphorylation of phospholamban requires the presence of both cAMP and free Ca^{2+}. Characterization and identification of phospholamban

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[1] Abbreviations: SR, sarcoplasmic reticulum; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PEG, poly(ethylene glycol); SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; Endo H, endo-β-*N*-acetylglucosaminidase H; 5′-FSO_2BzAdo, 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Me_2SO, dimethyl sulfoxide; ATPase, adenosinetriphosphatase; TCA, trichloroacetic acid.

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kinase therefore appear to be the first step in the understanding of this regulation in vivo.

Until now, phospholamban kinase was only poorly characterized. Le Peuch et al. (1982) have reported that phospholamban kinase is a membrane-bound enzyme which is distinct from glycogen phosphorylase *b* kinase. Plank et al. (1983) have investigated Ca^{2+} and calmodulin requirements of phospholamban kinase, while Louis and Maffitt (1982) studied the physical properties of the membrane-bound enzyme. The identity of phospholamban kinase among cardiac SR proteins is still unknown.

A phospholamban kinase activity was solubilized from the membrane, and the enzyme was partially purified. A characterization of the kinase was performed: determination of the optimum pH, requirement for Mg^{2+} and ATP, substrate specificity, and estimation of the molecular weight. Calmodulin target proteins as well as ATP binding proteins were detected along the purification steps in order to identify the candidates for being phospholamban kinase.

Materials and Methods

Materials

[γ-^{32}P]ATP and iodine-125 were purchased from Amersham. Endo-*N*-acetylglucosaminidase H from *Streptomyces plicatus* was purchased from Miles Laboratories. Octaethylene glycol *n*-dodecyl monoether ($C_{12}E_8$) was obtained from Nikko Chemicals Co. Ltd. (Tokyo, Japan). Diethylaminoethylcellulose (DEAE 52) was obtained from Whatman Biochemicals Ltd. (U.K.), Ultrogel ACA 34 was from LKB (France), and CNBr-activated Sepharose 4B was from Pharmacia (France) Ltd. 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine was synthesized according to the procedure of Wyatt and Colman (1977). 5′-[*p*-(Fluorosulfonyl)benzoyl][8-^{14}C]adenosine (50 mCi/nmol), 8-azido-[γ-^{32}P]ATP (10 Ci/mmol), and Enhance were purchased from New England Nuclear. Calmodulin was isolated from ram testis as described by Autric et al. (1980) and was coupled to CNBr-activated Sepharose 4B according to Klee and Krinks (1978). Phosphorylase *b* (Fischer & Krebs, 1958) and myosin regulatory light chains (Perrie & Perry, 1970) were purified to homogeneity from rabbit skeletal muscle. Smooth muscle myosin light chains were purified from hog bladder according to the procedure described by Hathaway and Haeberle (1983). Rabbit skeletal muscle glycogen synthase and bovine brain synapsin 1 purified as in Mac Guinness et al. (1983) were a generous gift from Prof. P. Cohen, Dundee (U.K.). Ram testis calmodulin and lectins from *Canavalia ensiformis* were iodinated by the iodogen technique (Salacinski et al., 1981) as previously described (Molla et al., 1985).

Methods

Miscellaneous Methods. Dodecyl sulfate (5–20% gradient) gel electrophoresis was performed according to Laemmli (1970). Electrophoreses were also carried out on 10% polyacrylamide gel according to Weber and Osborn (1969), Pharmacia molecular weight markers being used. Samples for electrophoresis were denatured in 0.25 M DTT, 2.5 M urea, 2.5% dodecyl sulfate, and 20 mM Tris-HCl, pH 6.8 at 4 °C. Radioactively labeled gels were dried and autoradiographed at −70 °C by using intensifying screen and Kodak AR films. Phosphopeptide mapping following limited proteolysis was carried out as in Cleveland et al. (1977) with *Staphylococcus aureus* V8 protease (Miles Lab.).

Protein concentrations were determined by the Coomassie blue technique using bovine serum albumin as standard (Spector, 1978). Phosphorylations by the catalytic subunit of cAMP-dependent protein kinase were performed as previously described (Molla et al., 1985).

Endo H Treatment of Sarcoplasmic Reticulum Vesicles. Removal of carbohydrate from sarcoplasmic reticulum glycoproteins was carried out with Endo H essentially as described by Campbell and Mac Lennan (1982). Briefly, cardiac SR vesicles (300 μg per sample) were first heated at 100 °C, for 5 min, in the presence of 0.5% (w/v) SDS, then cooled, and diluted (1 to 2) with a solution of 2 mM PMSF–25 mM sodium citrate, pH 5.5. Incubation was performed for 5 h at 37 °C, in either the presence or the absence of Endo H (5 milliunits). The reaction was stopped by addition of electrophoresis sample buffer.

Membrane Preparation. Canine cardiac SR vesicles were prepared from dog hearts arrested in diastole by pentobarbital injection essentially as in Le Peuch et al. (1979) slightly modified (Molla et al., 1983a). In order to remove remaining membrane-bound calmodulin, a supplementary wash in 50 mM Hepes buffer, pH 7.5, containing 10 mM EDTA was performed.

Solubilization of Phospholamban Kinase. Dog cardiac SR vesicles (7 mg/mL) were suspended in 2 mM EDTA, 5 mM β-mercaptoethanol, and 50 mM glycerophosphate, pH 7.2. Vesicles were hand-homogenized in the presence of $C_{12}E_8$ (10 mg/mL) and stored overnight at 4 °C. The suspension was then centrifuged at 100 000*g* for 45 min. The soluble proteins were precipitated by addition of 10% (w/v) poly(ethylene glycol) 6000. The resulting pellet was dissolved in buffer A [50 mM NaCl, 2.5 mM EDTA, 15 mM β-mercaptoethanol, 10% glycerol, 50 mM Tris-HCl, pH 7.4, and $C_{12}E_8$ (0.1 mg/mL)].

Enzyme Assay. The Ca^{2+}/calmodulin-dependent SR kinase activity was determined from the amount of [^{32}P]phosphate incorporated into either the membrane proteins or the exogenous substrate. In order to use phospholamban as exogenous substrate, SR vesicles were depleted in endogenous kinase activity by heat treatment at 80 °C for 5 min. The assay mixture contained 10 mM magnesium acetate, 0.5 mM [γ-^{32}P]ATP (150 cpm/pmol), 0.2 μM calmodulin, 50 mM Tris-HCl, pH 8.6, and either 0.1 mM $CaCl_2$ or 2 mM EGTA. The reaction, carried out at 30 °C, was initiated by addition of enzyme and terminated by cold TCA precipitation on Whatman 3 MM filter paper as described by Corbin and Reimann (1974).

Identification of Calmodulin-Binding Proteins and Glycoproteins in SDS–Polyacrylamide Gels. Cardiac SR calmodulin binding proteins and glycoproteins were detected after separation of the proteins in Laemmli slab gels through their reaction with ^{125}I-calmodulin and ^{125}I-concanavalin A, respectively.

Calmodulin gel overlay technique was preformed as previously described (Molla et al., 1985). Glycoproteins were detected by using a similar procedure. The gel was washed in 2-propanol, acetic acid, and H_2O, 25/10/65 (v/v), to remove SDS. Proteins were then submitted to denaturation prior to a renaturing step (Molla et al., 1983b). The gel was overlaid with buffer B (0.5 mM magnesium acetate and 0.2 M Tris-HCl, pH 7.5) supplemented with bovine serum albumin (10 mg/mL) and then soaked in buffer B containing ^{125}I-concanavalin (200 nM, 10^6 cpm/pmol, 100 mL per slab). Unbound concanavalin was removed by an extensive wash of the gel in buffer B. Gels were then stained by the Coomassie blue technique, dried, and submitted to autoradiography.

Results

Optimization of the Assay Conditions. The determination of the optimum pH of phospholamban kinase in SR vesicles

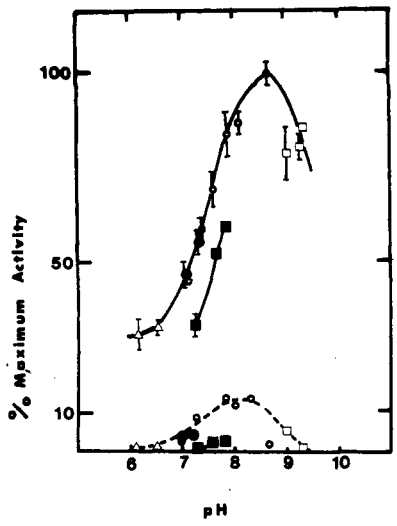

FIGURE 1: Determination of the optimum pH of phospholamban kinase. Cardiac SR vesicles (0.5 mg/mL) were incubated for 5 min as described under Methods but in the presence of the following buffers (50 mM) depending on the pH desired: Mes (△), pH 6.25–6.5; imidazole (●), pH 7–7.25; Hepes (■), pH 7.25–7.8; Tris (○), pH 7.0–8.5; boric acid (□), pH 9–9.25. The upper trace (—) represents the activity in the presence of Ca^{2+} while the lower trace (---) represents the activity in the absence of Ca^{2+}. Determinations were performed in triplicate. Error bars are indicated except when they are inferior to symbol size.

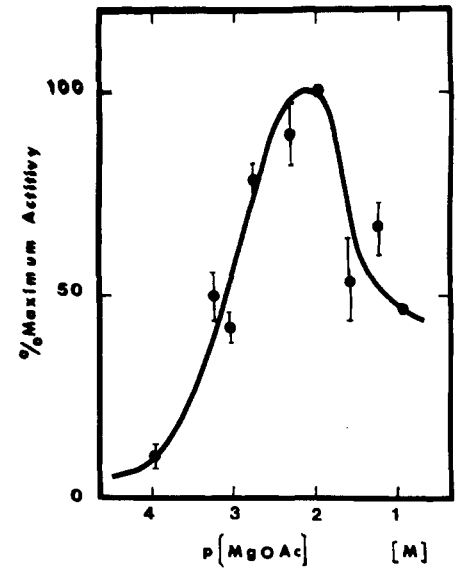

FIGURE 2: Effect of Mg^{2+} ions on phospholamban kinase activity. Cardiac SR vesicles (0.5 mg/mL) were incubated at 30 °C for 5 min, in the presence of 50 mM Tris-HCl, pH 8.6, under standard assay conditions described under Methods except that the Mg^{2+} concentration varies from 0.1 mM to 0.1 M. p(MgOAc) = −log (MgOAc). Values are the mean of two determinations.

is illustrated in Figure 1. The enzyme activity was studied in several buffers: Mes (pK_a = 6.15), imidazole (pK_a = 7.00), Hepes (pK_a = 7.55), Tris (pK_a = 8.30), and boric acid (pK_a = 9.24). Hepes buffer was found to decrease the kinase activity. At pH 7.5, phospholamban kinase activity was 1.5-fold higher in Tris than in Hepes buffer. The enzyme exhibits an optimum pH of 8.6, the activity falling off quite rapidly with rise or fall in pH. It is essentially inactive at pH below 6. The pH 6.8/8.2 activity ratio is 0.37. Such a value is not a consequence of the membrane environment, since similar results were obtained with the soluble enzyme (data not shown).

The effect of Mg^{2+} on the kinase activity is represented in Figure 2. Phospholamban kinase was assayed at pH 8.6 in the presence of 0.5 mM ATP as described under Methods. Maximal activity occurs at 10 mM Mg^{2+}, indicating that free Mg^{2+} is required in addition to MgATP. Inhibition of kinase activity occurs at higher Mg^{2+} concentrations.

Substrate Specificity. Treatment of cardiac SR vesicles with the mild detergent $C_{12}E_8$ [detergent/protein ratio of 1.43 (w/w)] allowed the solubilization of a kinase activity which is partially Ca^{2+} and calmodulin dependent. When such extract was precipitated by 6% (w/v) PEG, phospholamban kinase activity was detected in the pellet. This fraction exhibited a specific activity of 145 pmol incorporated into phospholamban min^{-1} mg^{-1}, in a Ca^{2+}-dependent manner. In this experiment, addition of PEG was limited at 6% (w/v), in order to obtain an active fraction totally free of phospholamban. The ability of the solubilized enzyme to phosphorylate various substrates was studied. Table I shows the relative Ca^{2+}/calmodulin-dependent phosphorylation rates for these substrates compared with skeletal glycogen synthase. In addition to phospholamban, synapsin-1, smooth muscle myosin light chain, and glycogen synthase were found to be good substrates for SR kinase while skeletal phosphorylase *b*, phosvitin, and casein were poorer substrates, and skeletal myosin light chain, histone mixture, and histone 2A showed little or no appreciable phosphate incorporation. Synapsin-1 contains multiple phosphorylation sites. Analysis of phosphorylated sites reveals that SR kinase phosphorylates synapsin-1 on an M_r 30 000 peptide generated by digestion with *Staphylococcus aureus* V8 protease (data not shown).

Table I: Substrate Specificity of Calmodulin-Dependent SR Kinase

substrate	concentration mg/mL	concentration μM	rel rates of phosphorylation (%)[a]
synapsin I	0.1	1.2	297
smooth muscle myosin P-light chain	0.8	40.0	130
glycogen synthase	0.4	4.6	100
phospholamban[b]	0.24	10.5	80
histone 3S	0.2	10.0	0
histone 2A	0.2	10.0	0
skeletal muscle myosin P-light chain	1.0	50.0	0
phosphorylase *b*	10.0	10.0	23
phosvitin	1.0	25.0	11
casein	1.0	42.0	13

[a] Rates of phosphorylation were measured as described under Methods. [b] The concentration of phospholamban was determined by taking into account that the proteolipid represents about 3% (w/w) of sarcoplasmic proteins (Capony et al., 1983).

In order to determine whether or not these phosphorylations were due to the same kinase, substrate competition experiments were performed. Kinetics of multiple substrate phosphorylations are represented in Figure 3. Comparison of parts A–C of Figure 3 indicates that addition of smooth muscle myosin light chains in the assay prevents phosphorylase *b* phosphorylation, as well as smooth muscle myosin light chain quenches phospholamban phosphorylation (see Figure 3, parts E and F). It can therefore be concluded that smooth muscle myosin light chain is a better substrate than phosphorylase *b*, thus confirming the data of Table I. No conclusion can be drawn from this experiment for phospholamban, since a sufficient concentration of the proteolipid could not be reached in the assay. However, the addition of phosphorylase *b* in cardiac SR vesicles was found to decrease phospholamban phosphorylation (Figure 4). These experiments carried out in the presence of 0.5 mM ATP reveal a competition between three substrates, smooth muscle myosin light chain, phosphorylase *b*, and phospholamban, suggesting thus that phospholamban

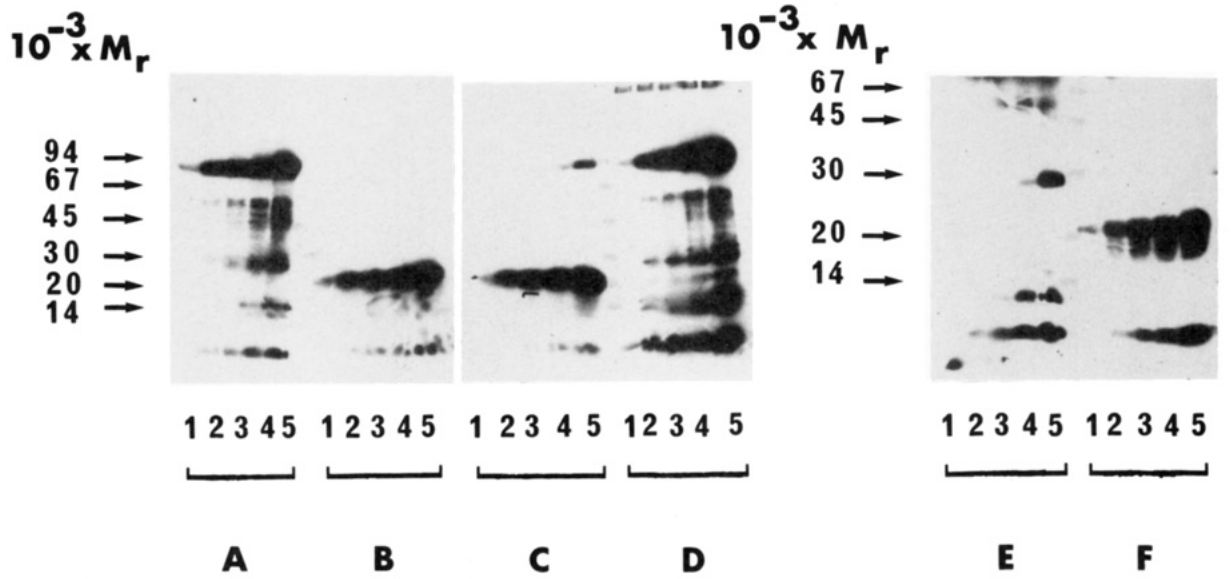

FIGURE 3: Substrate competition experiments. The 6% PEG pellet (40 μg) was assayed as described under Methods in the presence of 0.5 mM [γ-^{32}P]ATP (specific activity 300 cpm/pmol) and one or two substrates. An aliquot of the incubation medium was withdrawn after 2, 5, 8, 12, and 30 min of reaction and was diluted (1 to 2) with electrophoresis sample buffer. Samples were then submitted to either (5–20%) polyacrylamide gradient gels (parts A–D) or 15% polyacrylamide gels (parts E and F). The gels were dried and submitted to autoradiography. The autoradiograms corresponding to the kinetics of phosphorylation of phosphorylase (5 mg/mL), smooth muscle myosin light chain (0.8 mg/mL), and phospholamban (0.24 mg/mL) are represented in parts A, B, and E, respectively. The simultaneous phosphorylation of phosphorylase with either smooth muscle myosin light chain or phospholamban are shown in parts C and D, respectively, while part F represents the phosphorylation of phospholamban in the presence of smooth muscle myosin light chain. In lanes 1, 2, 3, 4, and 5 were loaded samples phosphorylated for 2, 5, 8, 12, and 30 min, respectively.

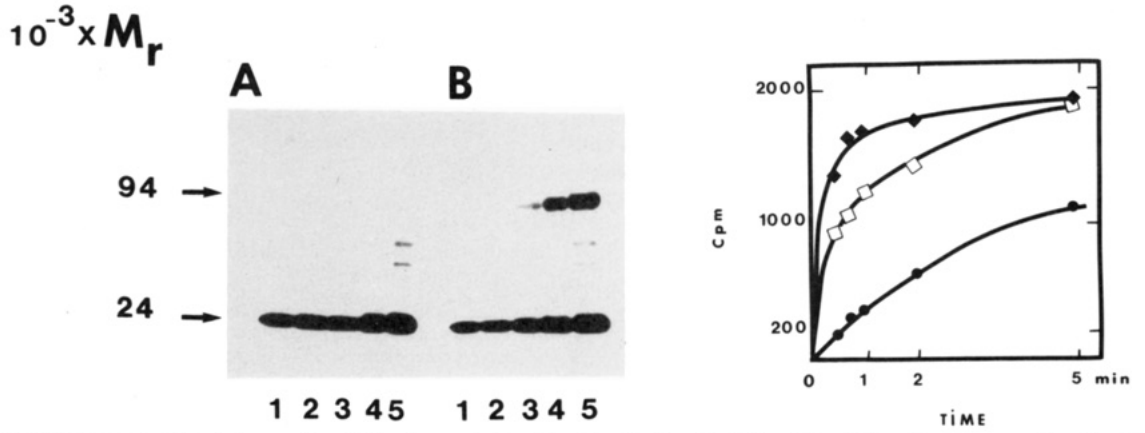

FIGURE 4: Substrate competition between phospholamban and phosphorylase *b*. Cardiac SR vesicles (300 μg) were assayed as described under Methods in the presence of 0.5 mM [γ-^{32}P]ATP (specific activity 300 cpm/mol) in either the presence (part B) or the absence (part A) of phosphorylase *b* (10 mg/mL). An aliquot of the incubation medium was withdrawn after 30 and 45 s and 1, 2, and 5 min of reaction and was diluted (1 to 2) with electrophoresis sample buffer. Samples were submitted to a 5–20% polyacrylamide gradient gel. The gel was dried and submitted to autoradiography. The corresponding autoradiogram is shown on the left part of this figure while the right part represents a quantification of this experiment. Small pieces of the dried gel corresponding to phospholamban and phosphorylase *b* were cut and counted for ^{32}P incorporation by using the Cerenkov radiation. The phosphorylation of phospholamban in either the presence or the absence of phosphorylase *b* is represented by (◊) and (♦), respectively, while (●) represents phosphorylase *b* phosphorylation.

kinase exhibits a broad substrate specificity.

Partial Purification of Cardiac Phospholamban Kinase. All procedures were carried out at 4 °C. All buffers contained 15 mM β-mercaptoethanol and 10% (v/v) glycerol.

(*a*) *DEAE-cellulose Chromatography.* Phospholamban kinase was extracted by $C_{12}E_8$, and the soluble fraction was precipitated by 10% (w/v) PEG as described under Methods. This fraction was applied to a 1 × 3 cm column of DEAE-cellulose, previously equilibrated with buffer A. Loading was performed for 1 h, in closed circuit. The column was then washed with buffer A and eluted with a linear salt gradient generated from 20 mL each of buffer A and buffer A containing 0.4 M NaCl. Figure 5 shows that a single peak of Ca^{2+}-dependent kinase activity was eluted at 0.104 M NaCl. The corresponding fractions were pooled and diluted 1.5-fold with buffer C (50 mM Tris-HCl, pH 7.5, 300 μM $CaCl_2$, 2 mM magnesium acetate, and 0.1 mg/mL $C_{12}E_8$) after EDTA neutralization by addition of 10 mM magnesium acetate and 300 μM $CaCl_2$.

(*b*) *Affinity Chromatography on Calmodulin–Sepharose.* The fraction obtained after chromatography on DEAE-cellulose was loaded, by overnight continuous application, onto a column of calmodulin Sepharose 4B (see Materials), previously equilibrated with buffer C. The column (1 × 2 cm) was washed with buffer C and then with buffer C complemented with 0.2 M NaCl. Finally, the kinase was eluted at a flow rate of 4 mL/h with buffer D (50 mM Tris-HCl, pH 7.5, 0.55 M NaCl, 5 mM EDTA, and 0.1 mg/mL $C_{12}E_8$). The purified enzyme was used within 4 h after the elution.

A summary of the purification is shown in Table II.

Table II: Purification of Cardiac SR Phospholamban Kinase[a,f]

step	total volume (mL)	total[b] proteins (mg)	total[c] act. (units)	sp act. (units/mg)	total[d] Ca^{2+}-dependent act. (units)	sp. act. (Ca^{2+}-EGTA) (unit/mg)	yield (%)	purification (x-fold)
SR vesicles	5.00	52.65	8108	154	7297	139	100	1
residue	5.00	28.10	6210	221	2981	84		
$C_{12}E_8$ extract	10.00	19.30	5751	298	3451	174	47	1.29
PEG pellet	5.43	13.41	3647	272	2918	218	40	1.57
DEAE-52 eluate	19.90	2.57	1350	526	1148	447	16	3.22
calmodulin–Sepharose eluate	3.57	0.13	871	6500[e]	783	5850	11	42.00

[a] Starting material: 60 g of canine heart. [b] Determined by the method of Spector (1978). [c] One unit is defined as that amount of enzyme which catalyzed the incorporation of 1 pmol of [^{32}P]phosphate into exogenous phospholamban/min under standard assay conditions (see Methods). [d] Ca^{2+}-dependent activity is the difference between the activity in the presence of Ca^{2+} minus the activity in the absence of Ca^{2+}. [e] This value is approximative since the protein concentration is very low, hardly quantified with precision by the Coomassie blue technique (Spector, 1978). [f] These values are the mean of five different preparations.

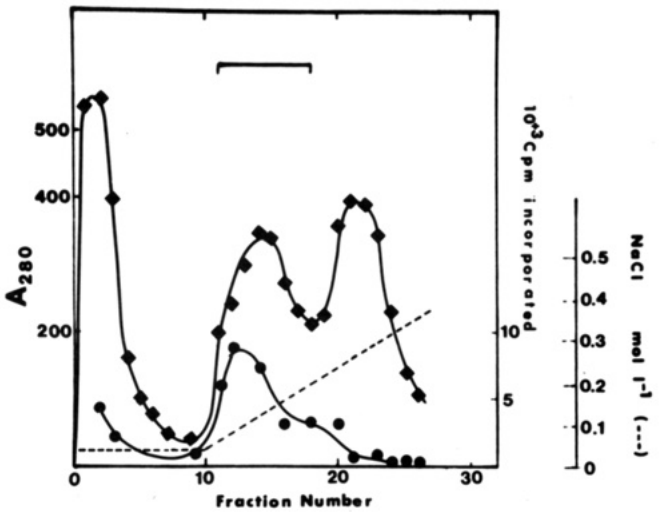

FIGURE 5: DEAE-cellulose ion-exchange chromatography of the 10% PEG pellet. The column was equilibrated with buffer A and eluted with a linear salt gradient (0.05–0.4 M NaCl) as described in the text at a flow rate of 10 mL/h. Fractions of 2.5 mL were collected and analyzed for their protein content (♦) and their salt concentration (---) by conductivity measurements. Aliquots (60 μL) were assayed for phospholamban kinase activity in either the presence or the absence of Ca^{2+}. The difference between these activities is represented (●). Fractions indicated by the bar were pooled and chromatographied on calmodulin–Sepharose.

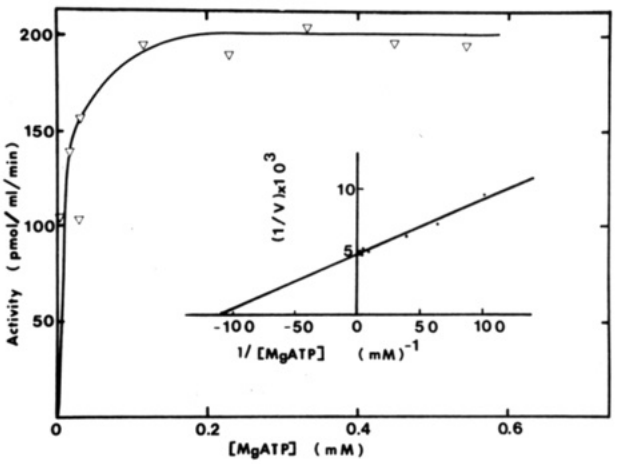

FIGURE 6: Determination of the K_m of partially purified phospholamban kinase for ATP. The fraction eluted from the calmodulin–Sepharose column was assayed for phospholamban kinase activity in the presence of various Mg^{2+} ATP concentrations, others conditions being as described under Methods. A kinetic experiment (5, 15, 25, and 40 min) was performed for each ATP concentration. The insert shows the Lineweaver–Burk representation of the data: linear regression was performed by the least-squares method. Intercept on abscissa −111 mM^{-1}.

Phospholamban kinase was purified approximately 42-fold with a yield of 11%. The purified fraction exhibits a specific activity of 6.5 nmol of [^{32}P]phosphate incorporated into phospholamban min^{-1} (mg of protein)$^{-1}$.

Determination of the K_m for ATP. Figure 6 illustrates the result of the determination of the K_m of partially purified phospholamban kinase for Mg^{2+}ATP. The dependence of the enzyme activity on Mg^{2+}ATP concentration follows Michaelis–Menten kinetics; analysis of the data by the least-squares method gives a K_m for Mg^{2+}ATP of 9 μM (see Figure 6 inset).

Substrate Specificity of the Partially Purified Kinase. In order to check whether or not the purified fraction exhibits a broad substrate specificity, phosphorylation experiments were conducted as shown in Figure 7.

Smooth muscle myosin light chain, phosphorylase *b*, and phospholamban are phosphorylated by the purified enzyme. In addition, the partially purified kinase phosphorylates a M_r 56 000 endogenous substrate in a Ca^{2+}/calmodulin-dependent manner (Figure 7, part C). The addition of smooth muscle myosin light chain decreases the M_r 56 000 endogenous phosphorylation, providing again an example of substrate competition (Figure 7, part D).

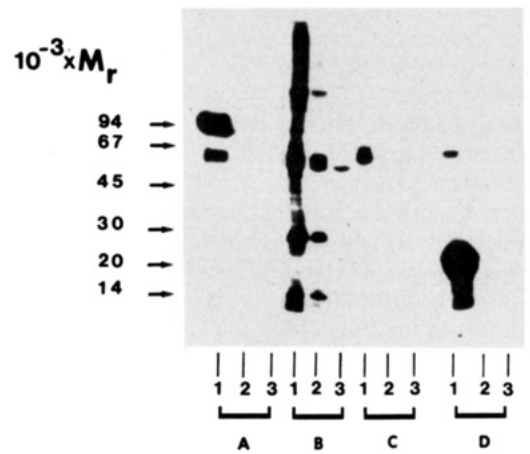

FIGURE 7: Phosphorylation of various substrates by the partially purified kinase. Phosphorylation experiments were performed for 15 min at 30 °C as described under Methods by using [γ-^{32}P]ATP as substrate in the presence of Ca^{2+} and either the presence (lane 1) or the absence (lane 2) of calmodulin and in the absence of Ca^{2+} (lane 3). The autoradiogram corresponding to phosphorylase phosphorylation is shown in part A, while parts B and D correspond to exogenous phospholamban and smooth muscle myosin light chain phosphorylation, respectively. Part C represents the phosphorylation of the purified kinase in the absence of exogenous substrate. Molecular weight markers were proteins in the Pharmacia low molecular weight kit.

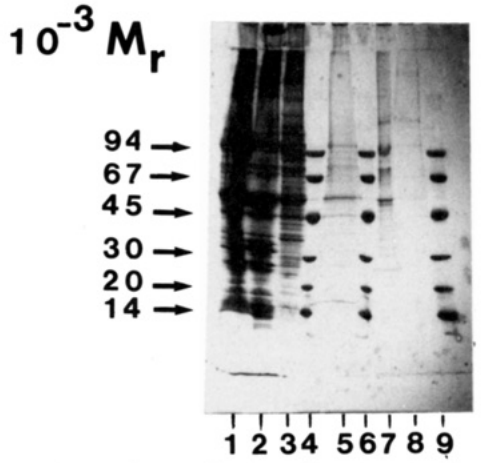

FIGURE 8: Dodecyl sulfate (0.1%)–(5–20%) polyacrylamide gradient gel electrophoretogram of the purification steps. Aliquots of proteins were diluted (1 to 2) by electrophoresis sample buffer and then submitted to electrophoresis. The gel was stained by Coomassie blue. Cardiac SR vesicles (300 μg), the resulting pellet (70 μg), the extract (100 μg), and the PEG pellet (40 μg) were loaded in lanes 1, 2, 3, and 5, respectively. The DEAE eluate (20 μg) and the calmodulin eluate (10 μg) were in lanes 7 and 8 while Pharmacia low molecular weights markers were in lanes 4, 6, and 9.

Gel Electrophoresis of the Purification Steps and Identification of ATP Binding Sites. The gel electrophoresis of the different purification steps (represented in Figure 8) reveals the presence of multiple proteins in the extract (lane 3). However, comparison of the extract (lane 3) with the nonsolubilized residue (lane 2) indicates that most of the proteins are extracted following the $C_{12}E_8$ treatment except for the low molecular weight proteolipids which are concentrated in the residue. The major proteins present in the DEAE eluate are the (Ca^{2+},Mg^{2+})-ATPase, the M_r 140000 protein, and a doublet of M_r 53000–56000. Traces of phospholamban are still present in this fraction. Analysis of the calmodulin–Sepharose eluate pattern reveals the presence of several faint bands, among which the M_r 140000, 105000, and 56000 proteins predominate.

Identification of ATP binding site in SR proteins was performed by using 5′-FSO_2BzAdo, an ATP analogue that irreversibly modifies adenine nucleotide binding sites (Colman et al., 1977); Hixson & Krebs, 1981). Preincubation of cardiac SR vesicles with 5′-FSO_2BzAdo was found to inhibit by about 50% phospholamban kinase activity (data not shown). 5′-FSO_2BzAdo is therefore a useful tool for labeling phospholamban kinase. The use of [^{14}C]-5′-FSO_2BzAdo revealed the presence of multiple adenine nucleotide binding sites in cardiac SR vesicles of M_r 140000, 105000, 94000, 84000, 55000, 46000, 42000, 35000, 31000, 26000, and 12000 (data not shown). Among them the M_r 105000 and 94000 entities can be ascribed to the (Ca^{2+},Mg^{2+})-ATPase and phosphorylase *b*, respectively, while the M_r 26000 and 12000 peptides comigrate with phospholamban. The M_r 140000 and 55000 proteins are sensitive to endo-β-glucosaminidase H and are therefore identical with the intrinsic glycoproteins described by Campbell et al. (1983). Furthermore, the use of azido-ATP indicated that, among adenine nucleotide binding proteins, the M_r 55000, 46000, 42000, and 35000 proteins are also ATP binding proteins. Labeling of the PEG pellet with [^{14}C]-5′-FSO_2BzAdo indicated that the M_r 84000, 55000 and 35000 proteins are still present in this fraction.

Catalog of Calmodulin Binding Proteins and Glycoproteins along the Purification Steps. The gel overlay technique with ^{125}I-labeled calmodulin allowed the detection of several calmodulin binding proteins of M_r 280000, 150000, 97000, 56000, 35000, and 24000 in cardiac sarcoplasmic reticulum (Figure 9A, lane 1; Molla et al., 1985). The M_r 150000, 56000, and 24000 target proteins are still present in the PEG pellet while only the M_r 56000 protein and traces of M_r 150000 calmodulin binding protein are detected in the calmodulin–Sepharose eluate (Figure 9A, lanes 6 and 7).

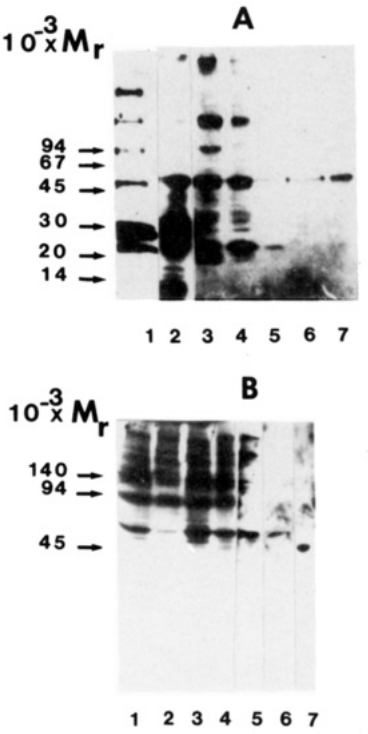

FIGURE 9: Detection of calmodulin binding proteins and glycoproteins. Proteins were submitted to a 5–20% polyacrylamide gradient slab gel. Calmodulin gel overlay technique was performed on part A while the glycoproteins were detected on part B. The corresponding autoradiograms are represented. SR vesicles (300 μg), the residue (70 μg), the $C_{12}E_8$ extract (100 μg), the 10% PEG pellet (120 μg), the DEAE eluate (20 μg), and the calmodulin–Sepharose eluate (10 μg) were loaded in lanes 1–6, respectively. The calmodulin–Sepharose eluate 4 times concentrated by 50% PEG precipitation was loaded in lane 7A. Pharmacia low molecular weight standards were loaded in lane 7B.

Glycoproteins were labeled in parallel by ^{125}I-concanavalin gel overlay technique. The corresponding autoradiogram represented in Figure 9B (lanes 1 and 4) indicated the existence of high molecular weight glycoproteins in addition to M_r 140000, 90000, 84000, and 55000 concanavalin binding proteins in cardiac SR vesicles as well in the PEG pellet and in the DEAE eluate. The M_r 55000 glycoprotein is the major concanavalin binding protein in the DEAE eluate (Figure 9B, lane 5) and is the only protein detected by this method in the calmodulin–Sepharose eluate (Figure 9B, lane 6).

Endogenous Phosphorylation by Phospholamban Kinase. When cardiac SR vesicles were phosphorylated in the presence of Ca^{2+} and calmodulin, the major phosphate acceptor was found to be phospholamban. However, when the PEG pellet was phosphorylated as shown in Figure 10 (lanes 1 and 2), ATP was also incorporated into a M_r 56000 protein and a M_r 94000 protein, in a Ca^{2+}/calmodulin-dependent manner. Phosphorylations were also examined by polyacrylamide gel electrophoresis according to Weber and Osborn (1969). In such a system, Ca^{2+}/calmodulin-dependent phosphate incorporations were detected for proteins of M_r 94000 and 57000, while phosphate was incorporated into a M_r 52000 protein whether or not Ca^{2+} was present (Figure 10, lanes 3–5).

Effect of Ca^{2+}/Calmodulin-Dependent Phosphorylation on Calmodulin Binding Proteins. As shown by the calmodulin

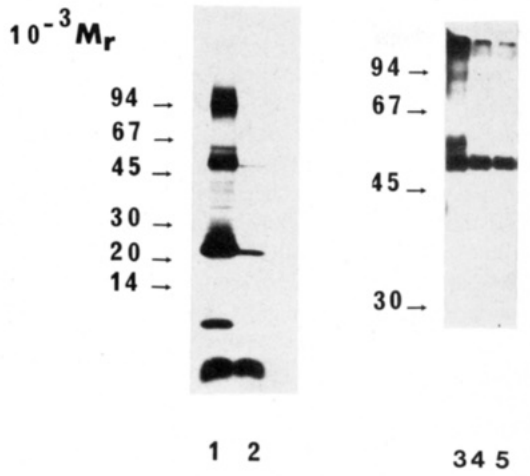

FIGURE 10: Autoradiogram of the endogenous Ca^{2+}/calmodulin-dependent phosphorylation. The purified fraction (PEG pellet) was phosphorylated as described under Methods, by using [γ-^{32}P]ATP as substrate, in the presence of Ca^{2+} and either the presence (lanes 1 and 3) or the absence (lanes 4) of calmodulin. Controls were performed in the presence of calmodulin and the absence of Ca^{2+} (lanes 2 and 5). The purified fraction was electrophoresed according to Laemmli (lanes 1 and 2) or to Weber and Osborn (lanes 3–5).

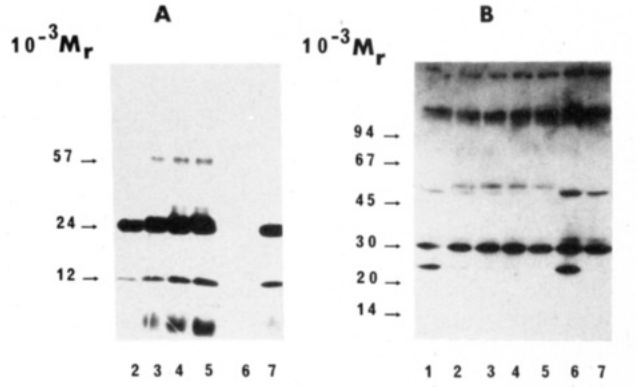

FIGURE 11: Effect of phosphorylations on cardiac SR calmodulin overlay pattern. Cardiac SR vesicles were phosphorylated by the endogenous Ca^{2+}/calmodulin-dependent protein kinase and the cAMP-dependent protein kinase as described under Methods. Experiments were performed in parallel with [γ-^{32}P]ATP and unlabeled ATP. All these samples were submitted to Laemmli slab gel electrophoresis. The ^{32}P-labeled part of the gel was submitted to autoradiography in order to monitor the extent of phosphorylation; the corresponding autoradiogram is shown in part A. Calmodulin gel overlay technique was performed on the nonradioactive part of the gel. The autoradiogram of ^{125}I-labeled calmodulin binding to target proteins is represented in part B. Unphosphorylated SR vesicles were loaded in sample lanes 1 and 6 while SR vesicles phosphorylated by phospholamban kinase for 1, 4, 10, and 30 min were loaded in lanes 2, 3, 4, and 5, respectively. SR vesicles phosphorylated by the catalytic subunit of cAMP-dependent protein kinase for 1 min were loaded in lane 7. In order to get better separation, the electrophoresis was stopped about 0.5 h after the dye reached the bottom of the gel.

overlay technique (Figure 11), cardiac SR calmodulin target proteins are modified upon phosphorylation by the endogenous Ca^{2+}/calmodulin-dependent protein kinase. As previously reported (Molla et al., 1985) calmodulin binding to phospholamban is modulated by phosphorylations. In addition, the Ca^{2+}/calmodulin-dependent phosphorylation modified the migration of M_r 56 000 calmodulin binding protein. The phosphorylated protein migrates slower than the dephospho entity. A doublet (M_r 56 000/57 000) is detected when SR vesicles are phosphorylated for 1 min, while the M_r 56 000 calmodulin binding protein has totally disappeared within 30 min of phosphorylation (Figure 11, lanes 1–6). Phosphorylation by the catalytic subunit of cAMP-dependent protein kinase does not alter the electrophoretic migration of this calmodulin binding protein (Figure 11B, lane 7).

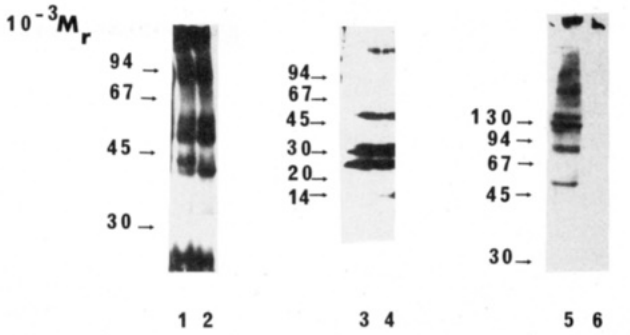

FIGURE 12: Endo H digestion of cardiac muscle sarcoplasmic reticulum proteins. Cardiac SR vesicles were solubilized in SDS and incubated in the presence (lanes 2, 4, and 6) or the absence (lanes 1, 3, and 5) of endo H as described under Methods. Cardiac SR vesicles phosphorylated in the presence of Ca^{2+}/calmodulin, as described under Methods, were submitted to electrophoresis according to Weber and Osborn (1969). The corresponding autoradiogram is shown in lanes 1 and 2. Unphosphorylated vesicles were analyzed by Laemmli slab gel electrophoresis, followed by either calmodulin overlay or glycoprotein overlay. ^{125}I-Calmodulin binding and ^{125}I-concanavalin A labeling are respresented in lanes 3–4 and 5–6, respectively.

Endo H Removal of Carbohydrate Chains. Endo H treatment was performed on cardiac SR vesicles previously phosphorylated by the endogenous Ca^{2+}/calmodulin-dependent protein kinase. Phosphorylated proteins were submitted to electrophoresis according to Weber and Osborn (1969). As shown in Figure 12 (lanes 1 and 2), calsequestrin, which is phosphorylated whether or not Ca^{2+} is present, is sensitive to Endo H digestion, while the M_r 57 000 and 52 000 phosphoproteins are not. Modification of calmodulin binding proteins following Endo H treatment was examined by calmodulin overlay. Cardiac SR vesicles calmodulin overlay pattern is not modified by Endo H digestion (Figure 12, lanes 3 and 4), while under the same conditions, the glycoprotein overlay pattern is drastically modified (see Figure 12, lanes 5 and 6).

DISCUSSION

Phospholamban, the cardiac SR proteolipid, is the substrate for several protein kinases, one of which is a Ca^{2+}/calmodulin-dependent protein kinase. This phospholamban kinase is only poorly characterized, probably as a consequence of the difficulties encountered with the purification of membrane-bound enzymes. In a first attempt directed toward the purification of phospholamban kinase, the enzyme lost its Ca^{2+} regulation upon detergent solubilization (Le Peuch et al., 1982). By using a mild nonionic detergent ($C_{12}E_8$), we succeeded in solubilizing a Ca^{2+}/calmodulin-dependent protein kinase. However, a Ca^{2+}-independent kinase activity is still present in the extract and represents about 40% of the total activity. The latter kinase was found to be sensitive to the heat-stable inhibitor of cAMP-dependent protein kinase, suggesting that the detergent treatment demasked the catalytic subunit of cAMP-dependent protein kinase (data not shown). The catalytic subunit is removed in part by PEG precipitation and does not bind to DEAE-cellulose. The solubilized Ca^{2+}/calmodulin-dependent phospholamban kinase was partially purified through two chromatography steps following a 10% PEG precipitation. The major purification step takes advantage of the affinity of the enzyme for calmodulin–Sepharose. The kinase binds quantitatively in the presence of Ca^{2+} ions and is eluted by EDTA. This indicates a Ca^{2+}-dependent interaction between the kinase and calmodulin similar to that reported in various calmodulin-dependent

processes [for review see Klee & Vanaman (1983)].

The purification procedure provides an approximately 42-fold purification with a yield of about 11%. The partially purified kinase exhibits a specific activity of 6.5 nmol min^{-1} (mg of protein)$^{-1}$. The final yield is about 0.13 mg of partially purified kinase per 60 g of ventricular tissue. Presently, such scarcity does not allow further purification.

Phospholamban kinase is absolutely dependent on Ca^{2+} and calmodulin. Phospholipids (phosphatidylserine and phosphatidylinositol) were unable to mimic the effect of calmodulin. Whereas phosphatidylserine can be substituted for calmodulin in the erythrocyte Ca^{2+}-ATPase activation (Roufogalis et al., 1982), it is inefficient toward phospholamban kinase. Addition of phosphatidylinositol to the assay increased by about 20% the phospholamban kinase activity, in the absence of calmodulin (data not shown). The apparent K_d of the kinase for calmodulin was shown to be dependent on the free Ca^{2+} concentration (Plank et al., 1983). In the presence of 100 μM Ca^{2+}, the apparent K_d of the kinase for calmodulin was determined to be 8 nM, regardless of the cAMP-dependent phosphorylation state of the vesicles (Molla et al., 1985). Louis and Maffitt (1982) have reported that phospholamban kinase activity increased as the pH was increased from pH 5.5 to 8.5, and their pH 6.8/8.2 activity ratio was determined to be 0.83. However, these authors used in the pH range studied Hepes buffer, which we have shown to be inhibitory (Figure 1). The pH dependence of phospholamban kinase was therefore examined by using a combination of several buffers. The optimum pH of phospholamban kinase is 8.6. This value is far from the physiological pH, but the possibility to reach such pH locally in the membrane is not unlikely. The pH 6.8/8.2 activity ratio is determined to be 0.37 and is therefore different from the value of 0.1 reported for glycogen phophorylase kinase (Cohen et al., 1981). This is additional evidence that although phospholamban kinase is able to phosphorylate phosphorylase *b*, it is distinct from glycogen phosphorylase kinase. Furthermore, whereas glycogen phosphorylase kinase and myosin light chain kinase exhibit relatively high K_m for ATP (about 200–400 μM) (Walsh et al., 1979; Nimmo & Cohen, 1974), the apparent K_m of phospholamban kinase for ATP is only 9 μM. The about 4 times higher value (37 μM) reported by louis and Maffitt (1982) may be explained by the fact that these authors are working with whole cardiac SR vesicles, where several ATP-binding and splitting proteins are present, especially the (Ca^{2+} + Mg^{2+})-ATPase. Other calmodulin-dependent protein kinases were found to exhibit low apparent K_m values for ATP in the micromolar range: synapsin-1 kinase II (Kennedy et al., 1983) and brain tubulin kinase (Goldering et al., 1983). The small quantities of available kinase makes a precise determination of the molecular weight difficult. However, a high molecular weight (M_r > 250 000) can be predicted for phospholamban kinase since the enzyme was eluted in the void volume from an Ultrogel ACA 34 chromatography (data not shown). Phospholamban kinase presents a broad substrate specificity, phosphorylating smooth muscle myosin light chain, synapsin-1, glycogen synthase, phosphorylase *b*, a M_r 56 000 endogenous substrate, and phospholamban. The possibility of a mixture of several kinases is unlikely since substrates were shown to compete in phosphorylation experiments. Calmodulin-dependent protein kinases from various tissues have been reported to present a broad substrate specificity. Comparison between glycogen synthase kinase and synapsin-1 kinase II reveals that these protein kinases share similar substrate and site specificity (Mac Guinness et al., 1983). They were also shown to exhibit immunological cross-reactivity (Woodgett et al., 1984). These kinases were proposed to be isoenzymes of a multifunctional Ca^{2+}/calmodulin-dependent protein kinase (Mac Guinness et al., 1983). Since, on the one hand, phospholamban kinase exhibits a broad substrate specificity and, on the other hand, phospholamban has been reported to be phosphorylatable by the brain Ca^{2+}/calmodulin-dependent kinase (Jett et al., 1984), the possibility that phospholamban kinase is similar to the multifunctional kinase must therefore be examined. Optimum pH and requirements for Mg^{2+} ions and ATP determined for phospholamban kinase are close to values reported for other broad substrate specificity enzymes. Similar ionic strengths elute both kinases from a DEAE-cellulose column. In addition, phospholamban kinase phosphorylates synapsin-1 on a 30 000-Da peptide and may thus share the same site specificity as the multifunctional enzyme (Mac Guinness et al., 1983). However, the relative phosphorylation rates of various substrates by phospholamban kinase are somewhat different from the values reported, for example, for glycogen synthase kinase (Mac Guinness et al., 1983). Moreover, phosphorylase *b* which is a rather poor substrate of phospholamban kinase has never been reported to be phosphorylatable by any isoenzyme of the multifunctional kinase. Such phosphorylation of phosphorylase *b* does not seem to be due to contaminating phosphorylase kinase since this phosphorylation decreases in the presence of smooth muscle myosin light chains and the addition of phosphorylase *b* in cardiac SR vesicles decreases phospholamban phosphorylation. Phospholamban kinase therefore shares several features of the multifunctional kinase but presents a different substrate specificity. It may be a distinct isoenzyme of the multifunctional calmodulin dependent protein kinase. The existence of different isoenzymes is also supported by the existence of two distinct protein kinases exhibiting broad substrate specificity in brain: synapsin-1 kinase II (Kennedy et al., 1983) and tubulin kinase (Goldering et al., 1983). Tubulin kinase phosphorylates tubulin, microtubule-associated proteins, and glycogen synthase but does not use synapsin-1 as substrate. Furthermore, Kloepper and Landt (1984) have reported the existence of a multifunctionnal kinase is bovine heart cytosol.

All these multifunctional kinases are reported to be high molecular weight enzymes composed of autophosphorylated subunits of M_r 50 000–60 000 (Goldering et al., 1983; Kennedy et al., 1983; Woodgett, et al., 1983). The autophosphorylation modifies the electrophoretic migration of the subunits (Goldering et al., 1983; Gorelick et al., 1983). In cardiac sarcoplasmic reticulum, several proteins migrating with such molecular weight had been reported: calsequestrin, a M_r 55 000 protein (Jones et al., 1981; Campbell et al., 1983), the intrinsic M_r 53 000 glycoprotein which binds ATP (Campbell et al., 1983), the M_r 56 000 calmodulin binding protein (Molla et al., 1985), and a M_r 56 000 phosphorylatable protein (Figure 7). Calsequestrin migrates as a M_r 44 000 species in neutral polyacrylamide gel electrophoresis (Weber & Osborn, 1969) and is sensitive to Endo H digestion. This protein, which undergoes phosphorylation whether or not Ca^{2+} is present (data not shown), binds neither calmodulin nor ATP. In addition, calsequestrin is absent from the PEG pellet; the possibility of this protein being the kinase is therefore unlikely. By contrast, the M_r 56 000 calmodulin target protein, the M_r 56 000 phosphorylatable protein, and the intrinsic glycoprotein are present in the active fractions and especially in the calmodulin–Sepharose eluate. Such data raise the possibility that the same protein exhibits altogether the calmodulin binding site, the ATP binding site, and the phosphorylation site. The

phosphorylation in the presence of Ca^{2+} and calmodulin promotes a slight modification in the electrophoretic migration of the M_r 56 000 calmodulin binding protein. It can therefore be concluded that the M_r 56 000 calmodulin binding protein is phosphorylated in the presence of Ca^{2+}/calmodulin. The calmodulin binding site and the phosphorylation site reside thus in the same entity. Such a protein shares several features in common with the subunits of the multifunctional kinases: similar molecular weight, phosphorylation in the presence of Ca^{2+}/calmodulin with a modification in the electrophoretic migration, and binding of calmodulin. However, the M_r 56 000 protein was found to be resistant to endo H while the M_r 53 000 glycoprotein was reduced to a M_r 49 000 protein. The intrinsic glycoprotein and the M_r 56 000 calmodulin binding protein are thus distinct. The intrinsic glycoprotein was never found to bind calmodulin either by the calmodulin overlay technique or by using an azido calmodulin derivative. This suggests that the glycoprotein by itself cannot be phospholamban kinase.

Taking together these data, it can therefore be proposed that phospholamban kinase is an isoenzyme of the multifunctionnal kinase. Phospholamban kinase is a high molecular weight enzyme composed of several subunits. The M_r 56 000 protein which is phosphorylated in the presence of Ca^{2+}/calmodulin, perhaps by autophosphorylation, is a possible candidate for being the calmodulin binding site of the kinase. The M_r 56 000 calmodulin binding protein was not found to bind ATP, but one cannot dismiss the possibility that several M_r 56 000 subunits interacting together exhibit an ATP binding site. Unitl now, the ATP binding site of the multifunctional kinase has never been defined. Another possibility can be that the M_r 56 000 subunit interacts with the M_r 53 000 intrinsic glycoprotein, the latter protein being the ATP binding site of the kinase. Further work will therefore be required to investigate these hypotheses and to identify phospholamban kinase.

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Registry No. ATP, 56-65-5; phospholamban kinase, 85638-42-2; glycogen synthase, 9014-56-6.

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Purification and Biochemical Characteristics of Two Distinct Human Interleukins 1 from the Myelomonocytic THP-1 Cell Line

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ABSTRACT: An effective induction protocol for the production of interleukin 1 (IL 1) by human myelomonocytic cell line (THP-1) cells was developed, and two biochemically distinct human IL 1 peptides were purified. Lipopolysaccharide, silica, and hydroxyurea by themselves did not induce IL 1 production, but these three stimulants in combination had a synergistic effect on the production of IL 1 by THP-1 cells. A 17-kilodalton (kDa) form of human IL 1 with a p*I* of 7.0 (IL 1-β) was purified to homogeneity by sequential chromatography on DEAE-Sephacel, Sephacryl S-200, CM high-performance liquid chromatography (HPLC), and hydroxyapatite HPLC. The recovery of IL 1-β activity was 45%, and the specific activity was 2.3×10^7 units/mg. Both IL 1-β and a second 17-kDa IL 1 moiety with a p*I* of 5.0 (IL 1-α) were also extracted from stimulated THP-1 cells and purified to homogeneity by sequential chromatography on Sephacryl S-200, ion exchange HPLC, and hydroxyapatite HPLC. The recovery of IL 1-β from cell extracts was 5.6%, and the specific activity was 3×10^7 units/mg. In contrast, only 0.85% of IL 1-α was recovered with a specific activity of 5.3×10^7 units/mg. The amino acid sequence of the amino-terminal end and amino acid composition of purified IL 1-β obtained from both culture supernatants and cell extracts of THP-1 cells showed complete identity with those of IL 1-β from culture supernatants of normal human monocytes, suggesting that normal human monocytes and THP-1 cells produce identical intracellular human IL 1-β and release the same activity into the culture supernatant. The amino composition of IL 1-α in cells extracts of THP-1 cells was noticeably different from that of IL 1-β but closely resembled the predicted amino acid composition of the 17-kDa C-terminal portion deduced from the published cDNA nucleotide sequence of IL 1-α, further supporting the existence of two distinct human IL 1 genes. In conclusion, the purification and biochemical characterization of two distinct human IL 1 moieties (IL 1-α and IL 1-β) from a human myelomonocytic cell line establish that this leukemic cell line produces two distinct IL 1 moieties that are biologically and biochemically identical with the products of normal human monocytes. Consequently, THP-1 cells provide a good source of IL 1 that is identical with that produced by normal human monocytes.

We have previously reported the purification of human IL 1-β[1] (p*I* 7.0, molecular mass 17 kDa) from culture supernatants of normal human peripheral blood adherent cells (monocytes) and that such purified IL 1-β has thymocyte comitogenic activity, fibroblast proliferation activity, acute phase protein inducing activity, and endogenous pyrogen activity (Matsushima et al., 1985a). The amount of purified IL 1-β that could be obtained from normal human monocytes was very limited. For example, 5 L of supernatants from $\sim 5 \times 10^9$ cultured adherent cells that were derived from 25×10^9 peripheral blood mononuclear cells yielded only 4 μg of IL 1 (Matsushima et al., 1985a). But, this did not suffice for studies of the primary structure and possible posttranslational modifications of human IL 1. Although the purification of human IL 1 from human cell lines could potentially provide large amounts of IL 1, only a few papers report low levels of IL 1 production by human cell lines (Palacios et al., 1982; Krakauer & Oppenheim, 1983). Furthermore, March et al. have recently cloned two distinct (acidic and neutral) human IL 1 (IL 1-α and IL 1-β) cDNA coding for two distinct human IL 1 proteins (March et al., 1985), which probably correspond with the p*I* 5.0 and 7.0 peaks of natural human IL 1 activity detected by isoelectrofocusing (Matsushima, 1985b). However, so far, there is no direct evidence of the existence of human IL 1-α in either culture supernatants or cell extracts. We have, therefore, developed an efficient induction protocol

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[1] Abbreviations: IL 1, interleukin 1; FCS, fetal calf serum; LPS, lipopolysaccharide; HU, hydroxyurea; D-PBS, Dulbecco's phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; NaPB, sodium phosphate buffer; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; 2-ME, 2-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride.

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