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Biochemistry

Substrate proteins for calmodulin-sensitive and phospholipid-sensitive Ca^{2+}-dependent protein kinases in heart, and inhibition of their phosphorylation by palmitoylcarnitine

(ischemic heart/cyclic nucleotides/subcellular fractions)

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ABSTRACT **At least two substrate proteins for phospholipid-sensitive Ca^{2+}-dependent protein kinase and at least six substrates for calmodulin-sensitive Ca^{2+}-dependent protein kinase were identified in the cytosol of the guinea pig heart. In the particulate subfractions enriched in nuclei, mitochondria, microsome, or plasma membrane, no substrates for the phospholipid-sensitive enzyme were demonstrated but at least four substrates for the calmodulin-sensitive enzyme were identified. The present studies suggest that phospholipid, acting independently of calmodulin, is likely to be involved in the regulation of Ca^{2+}-dependent protein phosphorylation in the heart. Phosphorylation of endogenous substrates for the two enzyme systems was effectively inhibited by palmitoylcarnitine. When histone was used as exogenous substrate, the carnitine ester inhibited the cardiac phospholipid-sensitive Ca^{2+}-dependent protein kinase but not the cardiac cyclic AMP-dependent and cyclic GMP-dependent protein kinases. It is suggested that inhibition of the Ca^{2+}-dependent phosphorylation of cardiac proteins, regulated by either phospholipid or calmodulin, is probably related in part to the great increase in this fatty acid metabolic intermediate in the ischemic heart.**

The Ca^{2+}-dependent phosphorylation of proteins has been recognized as a major regulatory mechanism of biological processes. Protein kinases that are activated by Ca^{2+} include phosphorylase kinase (1–3), myosin light chain kinase (4–7), glycogen synthase kinase (8), and those responsible for phosphorylation of numerous endogenous proteins in the membrane (9–12) and soluble (12, 13) fractions from the brain and adipocyte. All of these Ca^{2+}-dependent enzymes require the presence (either as an enzyme subunit or cofactor) of calmodulin, a Ca^{2+}-binding protein (14) that is also involved in various other Ca^{2+}-requiring reactions or processes (15).

Takai *et al.* (16) have identified and partially purified from the rat brain a new species of Ca^{2+}-dependent protein kinase (Ca-PKase), which requires phospholipid (such as phosphatidylserine) instead of calmodulin as a cofactor. We have recently reported (17) a widespread occurrence of this phospholipid-sensitive Ca-PKase in various tissues of animals of different phyla. We have also shown the presence in rat and guinea pig cerebral cortex of its substrate proteins which are different from those for the calmodulin-sensitive species of Ca-PKase also seen in the same tissue (12). In view of the importance of Ca^{2+} in cardiac function and the potential role of phospholipid as a biological regulator, studies dealing with this newly recognized Ca^{2+} target enzyme system seem warranted.

We report here the subcellular distribution of endogenous substrate proteins for phospholipid-sensitive Ca-PKase and those for calmodulin-sensitive Ca-PKase. We also report here inhibition of the two Ca-PKase systems by palmitoylcarnitine, an intermediate of long-chain fatty acid metabolism that has been shown to accumulate to high (millimolar) concentrations in the ischemic heart (18–20).

EXPERIMENTAL PROCEDURES

Materials. Phosphatidylserine (bovine brain), lysine-rich histone (type III-S), histone (mixture, type II), and DL-palmitoylcarnitine were purchased from Sigma.

Methods. The procedures for the preparation of the nuclei (21), mitochondria (22), microsome (23, 24), and plasma membrane (25) fractions from guinea pig hearts were as described by others. In some experiments, the heart was homogenized in 10 vol of 0.25 M sucrose/25 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/50 mM 2-mercaptoethanol, and the homogenate was centrifuged at 105,000 × *g* for 60 min. The resulting supernatant and pellet, designated cytosol and total particulate, were used directly as the sources of protein kinases and their substrates. Phospholipid-sensitive Ca-PKase was purified >10,000-fold (about 90% homogeneous) from the bovine heart extract through the steps of ammonium sulfate fractionation and chromatography on DEAE-cellulose, controlled-pore glass, Sephadex G-200, and phosphatidylserine-Affigel 102 columns. The Ca-PKase activity was assayed essentially as described (12, 17). In brief, the incubation mixture (0.2 ml) contained 5 μmol Tris·HCl (pH 7.5), 2 μmol $MgCl_2$, 40 μg of lysine-rich histone, 5 μg of phosphatidylserine, with or without 0.1 μmol of $CaCl_2$, 0.05 μmol of ethylene glycol bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid (EGTA), and 1 nmol of [γ-^{32}P]ATP containing about 1.1×10^6 cpm. Cyclic AMP-dependent protein kinase (A-PKase) (26) and cyclic GMP-dependent protein kinase (G-PKase) (26) were also prepared from the bovine heart extract; their activities were assayed by described methods (26, 27) with mixed histone as substrate. The methods for phosphorylation of cardiac proteins, electrophoresis on $NaDodSO_4$/polyacrylamide gel, and subsequent autoradiography of the phosphoproteins were as described for the brain (12), with 5–30 μM [γ-^{32}P]ATP. Calmodulin was purified to apparent homogeneity from the rat brain extract by the fluphenazine affinity method of Charbonneau and Cormier (28). [γ-^{32}P]ATP was prepared by the method of Post and Sen (29), and protein was determined by the method of Lowry *et al.* (30).

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Abbreviations: Ca-PKase, Ca^{2+}-dependent protein kinase; A-PKase, cyclic AMP-dependent protein kinase; G-PKase, cyclic GMP-dependent protein kinase; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetate; IC_{50}, concentration causing inhibition by 50%.

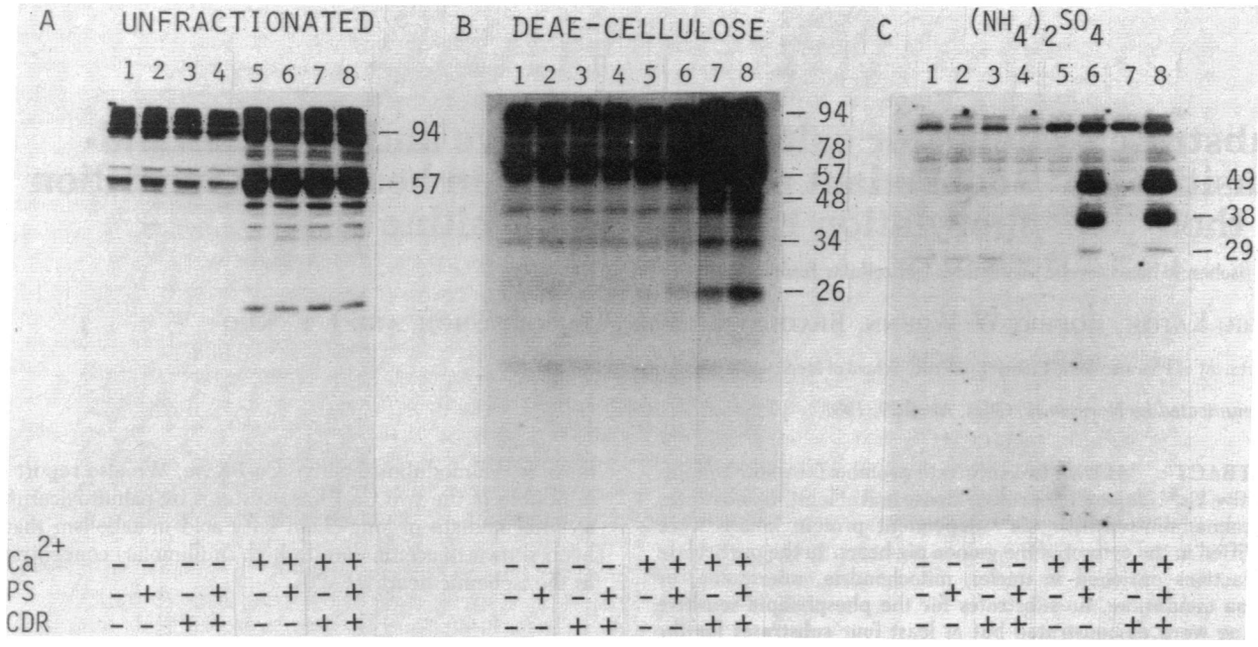

FIG. 1. Autoradiograms showing phosphorylation of proteins from the guinea pig heart cytosol under various incubation conditions. (*A*) Unfractionated cytosol; (*B*) protein peak eluted from DEAE-cellulose column with 100 mM ammonium sulfate; (*C*) fraction precipitated with ammonium sulfate (35–70%, wt/vol). Phosphorylation of endogenous proteins was carried out as described (12), in 0.2 ml, at 30°C for 5 min, in the presence or absence of $CaCl_2$ (0.1 μmol; final concentration, 500 μM), phosphatidylserine (PS, 5 μg), and calmodulin (CDR, 2 μg), as indicated. EGTA (250 μM) was included in all cases in order to minimize phosphorylation in the absence of added $CaCl_2$. The separating gel was 12% acrylamide containing 0.1% $NaDodSO_4$. The amount of protein applied in each lane was 30 μg. Molecular weights are shown $\times 10^{-3}$.

RESULTS

Although Ca^{2+} stimulated phosphorylation of endogenous proteins (notably 94,000- and 57,000-dalton species) in the whole cytosol of the guinea pig heart, the Ca^{2+}-dependent reaction was not augmented by either calmodulin or phospholipid (Fig. 1*A*). The cytosol was chromatographed on DEAE-cellulose, in order to remove possible substances (such as calmodulin and phosphoprotein phosphatases) that may render calmodulin-sensitive and phospholipid-sensitive Ca^{2+}-dependent phosphorylation of endogenous proteins undemonstratable. The protein peak eluted with 100 mM ammonium sulfate was found to contain both the enzyme and substrates for the calmodulin-sensitive system; calmodulin was eluted with 450 mM salt (not shown). Phosphorylation and autoradiography of the protein peak eluted with 100 mM salt revealed at least six substrate proteins (94,000, 78,000, 57,000, 48,000, 34,000, and 26,000 daltons) that were specific for the calmodulin-sensitive Ca-PKase (Fig. 1*B*); no substrates for the phospholipid-sensitive enzyme, however, were detected in the same protein peak. In the fraction of cytosol precipitated with $(NH_4)_2SO_4$ (35–70%, wt/vol) we identified two major substrates (49,000 and 38,000 daltons) for the phospholipid-sensitive enzyme (Fig. 1*C*); no substrates for calmodulin-sensitive Ca-PKase were detected in this fraction.

In the total unfractionated particulate of the guinea pig heart, we noted four substrates (92,000, 59,000, 57,000, and 16,000 daltons) specific for calmodulin-sensitive Ca-PKase; none was demonstrated for the phospholipid-sensitive enzyme (not shown). The occurrence and distribution of substrates were further investigated in subcellular fractions. The 16,000-dalton substrate was found in the nuclear and mitochondrial fractions (Fig. 2). Phosphorylation of the 44,000-dalton protein in the mitochondria was augmented by calmodulin, in the absence or presence of added $CaCl_2$. Its phosphorylation in the presence of added $CaCl_2$, however, was less, suggesting that Ca^{2+} may activate dephosphorylation of this phosphoprotein.

Three major substrates (92,000, 57,000, and 16,000 daltons) and four minor substrates (ranging from 38,000 to 57,000 daltons) were demonstrated in the microsomal fraction (Fig. 3*A*). Four major substrates (92,000, 59,000, 57,000, and 16,000 daltons) were observed for the plasma membrane fraction (Fig. 3*B*). As in the microsomal fraction, the 16,000-dalton protein appeared to be the prominent substrate in the sarcolemma and its phosphorylation was absolutely dependent upon the combined presence of calmodulin and Ca^{2+}.

In all cases, not a single substrate specific for the phospholipid-sensitive Ca-PKase was demonstrated in any of the four particulate subfractions (Figs. 2 and 3), as in the case for the total unfractionated particulate (not shown), of the guinea pig heart. The subcellular fractions used in the present studies were greatly enriched in the respective organelles, as indicated by the fact that 90% of the total Ca^{2+},Mg^{2+}-ATPase was found in the microsomal fraction; 88% of Na^+,K^+-ATPase was in the sarcolemmal fraction; and more than 50% of succinic dehydrogenase was in the mitochondrial fraction. We also noted that the cytosol contained 72% of the total phospholipid-sensitive Ca-PKase activity, the sarcolemmal fraction contained 9%, the microsomal fraction contained 18%, and the mitochondrial and nuclear fractions contained 1%.

It has been shown that palmitoylcarnitine is increased (from 0.4 mM to 2 mM) during myocardial ischemia (18–20) and that the fatty acid metabolic intermediate alters membrane properties (18–20, 31) and inhibits Na^+,K^+-ATPase and Ca^{2+},Mg^{2+}-ATPase (24) of the heart. In the present studies, we found that palmitoylcarnitine (70 μM) nearly completely inhibited the cardiac phospholipid-sensitive Ca-PKase but essentially had no effects on the cardiac A-PKase and G-PKase (Table 1). In all cases, these protein kinases were assayed with histone as substrate. Phosphorylation of endogenous proteins from the heart cytosol, similar to those shown in Fig. 1*C*, catalyzed by phospholipid-sensitive Ca-PKase was also inhibited by the carnitine ester (Fig. 4*B*). It also similarly inhibited phosphorylation of substrate proteins catalyzed by calmodulin-sensitive Ca-PKase

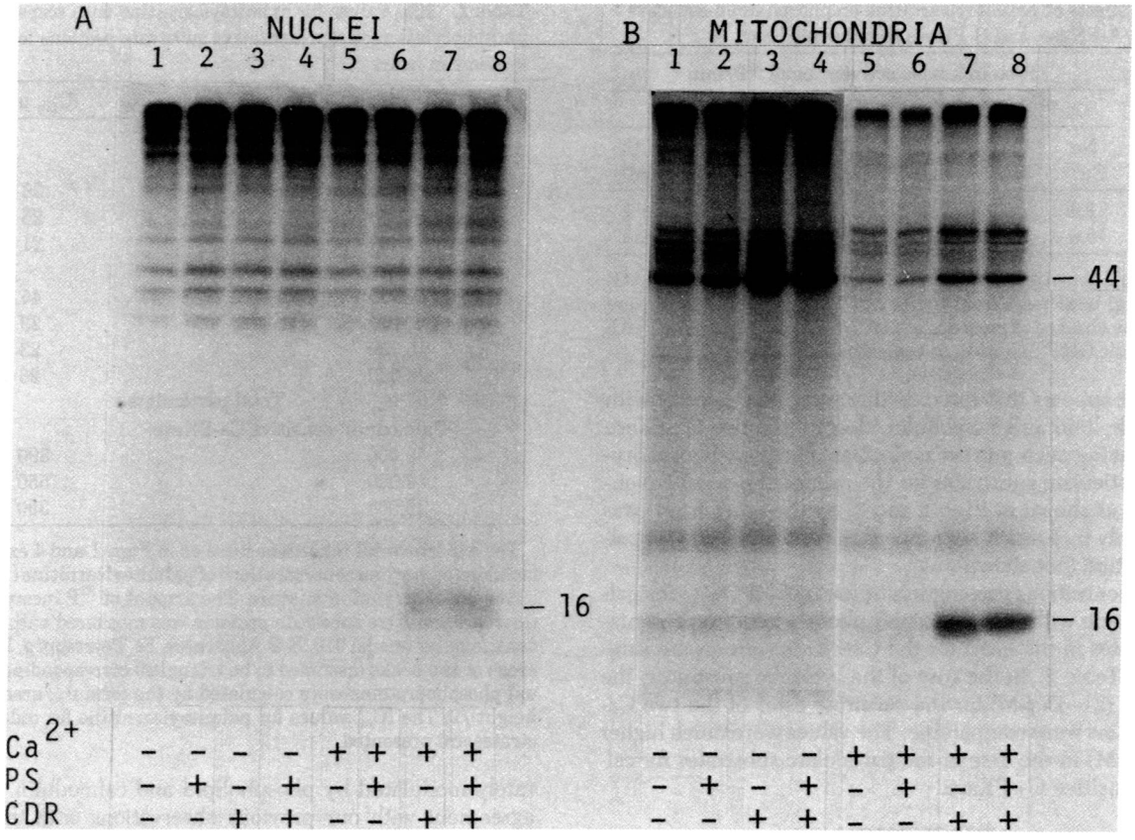

FIG. 2. Autoradiograms showing the effects of Ca^{2+}, phosphatidylserine (PS), and calmodulin (CDR) on phosphorylation of proteins in nuclei (*A*) and mitochondria (*B*) from the guinea pig heart. The experimental conditions and procedures were as in Fig. 1. The amount of protein applied to each lane was 15 μg for nuclei (*A*) and 30 μg for mitochondria (*B*).

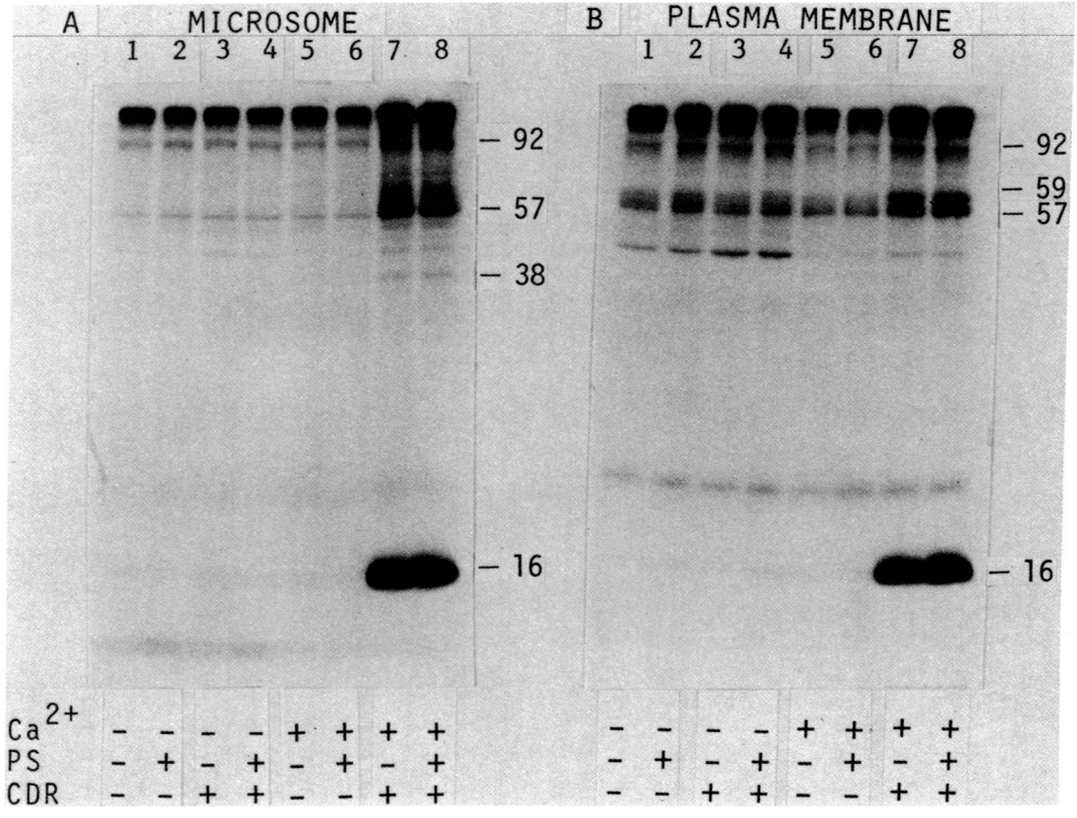

FIG. 3. Autoradiograms showing the effects of Ca^{2+}, phosphatidylserine (PS), and calmodulin (CDR) on phosphorylation of proteins in microsomes (*A*) and plasma membrane (*B*) from the guinea pig heart. The experimental conditions and procedures were as in Fig. 1. The amount of protein applied to each lane was 30 μg.

Table 1. Effects of palmitoylcarnitine on phospholipid-sensitive Ca-PKase, A-PKase, and G-PKase from bovine heart

Palmitoylcarnitine, μM	Protein kinase activity, pmol ^{32}P/min					
	Ca-PKase		A-PKase		G-PKase	
	No Ca^{2+}	With Ca^{2+}	No cAMP	With cAMP	No cGMP	With cGMP
0	1.4	8.2	0.7	5.3	0.9	11.1
70	0.6	1.2	1.2	5.4	0.9	9.8

Phospholipid-sensitive Ca-PKase (0.1 μg), A-PKase (5 μg), and G-PKase (12 μg) used were from the bovine heart. The enzymes were assayed in the absence or presence of $CaCl_2$ (500 μM), cyclic AMP (0.5 μM), and cyclic GMP (0.5 μM), as indicated.

(Fig. 4A). It appears that the carnitine ester may interact with both phospholipid and calmodulin, despite the two Ca-PKase cofactors having such a great molecular diversity. Phosphorylation of particulate substrates for the calmodulin-sensitive enzyme, such as shown in Figs. 2 and 3, on the other hand, was inhibited only by a much higher concentration (500 μM) of palmitoylcarnitine (not shown).

The concentrations causing inhibition by 50% (IC_{50}) for palmitoylcarnitine with respect to phosphorylation of representative substrates in the heart for the Ca-PKase systems are summarized in Table 2. In the case of the cytosolic substrates, the IC_{50} values (21–45 μM) for the carnitine ester of the two Ca-PKase systems were comparable. The values were much higher (390–550 μM) in the case of the particulate substrates for calmodulin-sensitive Ca-PKase.

Table 2. IC_{50} values for palmitoylcarnitine with respect to phosphorylation of representative substrate proteins for Ca-PKase systems in heart

Substrate protein, daltons	IC_{50}, μM
Cytosol	
Phospholipid-sensitive Ca-PKase:	
49,000	36
38,000	25
29,000	21
Calmodulin-sensitive Ca-PKase:	
78,000	45
57,000	29
48,000	23
26,000	26
Total particulate	
Calmodulin-sensitive Ca-PKase:	
59,000	390
57,000	550
16,000	390

The experimental conditions were as in Figs. 1 and 4 except for the inclusion of varying concentrations of palmitoylcarnitine (12–230 μM) in the phosphorylation mixture. The amount of ^{32}P incorporated into the representative substrate proteins was measured with a scanning densitometer (model 910, E-C Apparatus, St. Petersburg, FL), and the areas of the peaks (assumed to be triangles) corresponding to individual phosphoproteins were calculated by the formula: area = (base × height)/2. The IC_{50} values for palmitoylcarnitine for individual substrates are presented.

DISCUSSION

The present studies clearly demonstrate that Ca^{2+}-dependent phosphorylation of endogenous proteins in the heart is separately modulated by phospholipid and calmodulin. This is in agreement with our previous observations with the cerebral cortex (12). Due to the possibility that the standard experimental conditions used are not necessarily optimal for all fractions and Ca-PKase systems, the demonstrated presence of sub-

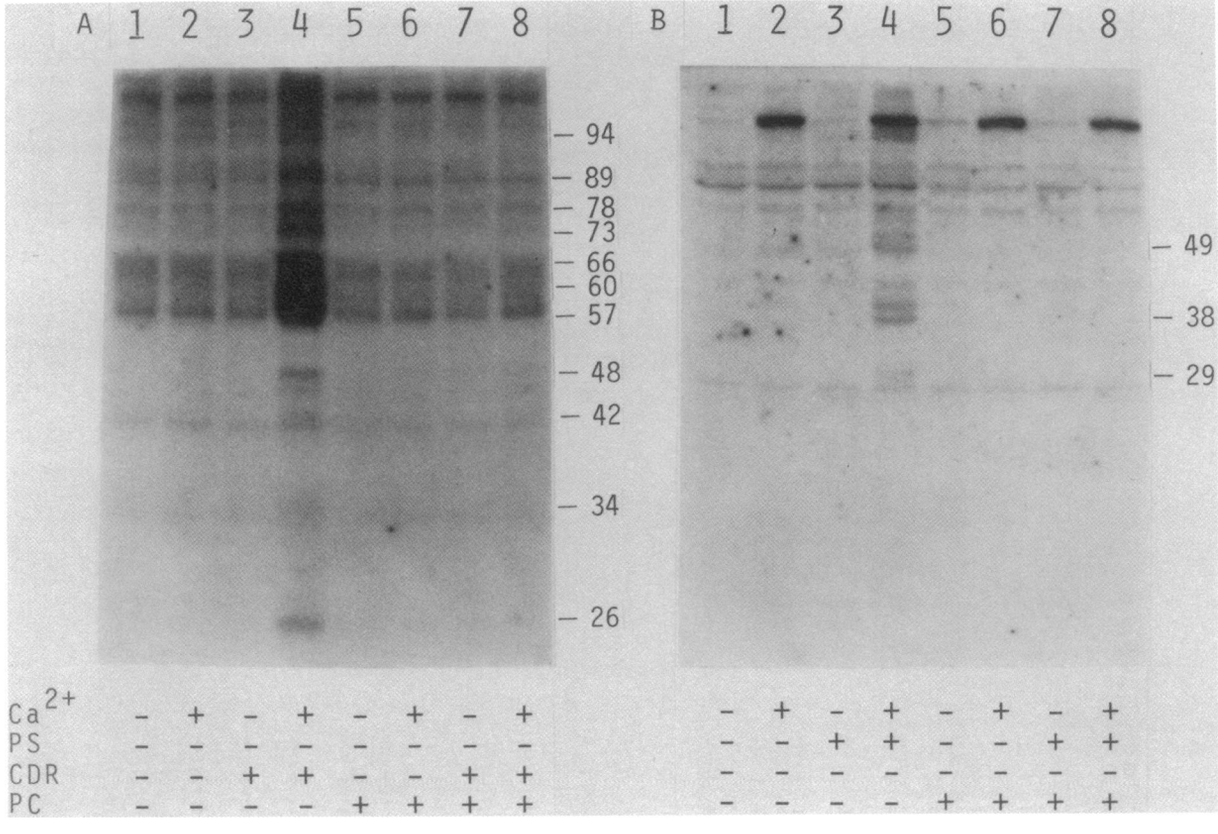

FIG. 4. Autoradiograms showing inhibition by palmitoylcarnitine of phosphorylation of substrate proteins in the heart cytosol. The experimental conditions were as in Fig. 1, except for the inclusion of palmitoylcarnitine (115 μM) in some incubations. (*A*) Calmodulin-sensitive Ca-PKase system (DEAE-cellulose fraction as in Fig. 1*B*); (*B*) phospholipid-sensitive Ca-PKase system (ammonium sulfate fraction, 35–70%, as in Fig. 1*C*).

strates may only be regarded as the minimal numbers and amounts of the total substrates that may be present. It should be noted, however, that we have also carried out phosphorylation experiments with higher concentrations (300 and 1000 μM) of [γ-^{32}P]ATP and found that 4–30 μM ATP [in the present and previous studies (12)] was optimal for demonstration of the phospholipid-sensitive, and possibly the calmodulin-sensitive, Ca^{2+}-dependent phosphorylation of endogenous proteins in the cytosolic and particulate fractions. We have determined recently that the purified cardiac phospholipid-sensitive Ca-PKase has a K_m for ATP of about 4 μM (unpublished data).

The cardiac substrate proteins observed in the present studies appeared to be different from myosin P light chain (20,000 daltons), the substrate for myosin light chain kinase (4–7), and the membrane endogenous substrates for A-PKase previously reported by others which include phospholamban at 22,000 daltons (32), A protein at 36,000 daltons, and B protein at 27,000 daltons (33, 34). The following issues are clearly in need of clarification before a biological significance of the present findings can be established: (*a*) the identities and functional roles of substrate proteins, either cytosolic or particulate, for the two Ca-PKase systems; (*b*) possible modifications in the properties of the substrate proteins upon phosphorylation or dephosphorylation; (*c*) the possible functional relationships between the phospholipid-sensitive and calmodulin-sensitive Ca-PKase systems, and possible interactions among Ca^{2+}, cyclic AMP, and cyclic GMP at the level of protein phosphorylation.

Because palmitoylcarnitine binds to the membranes due to its hydrophobic nature (24), the amounts and proportions of the carnitine ester that remain free are not clear. Nevertheless, the present observations that palmitoylcarnitine inhibited phosphorylation of histone by phospholipid-sensitive Ca-PKase (Table 1) and of endogenous proteins by the two Ca-PKase systems (Fig. 4 and Table 2), but not the activities of A-PKase and G-PKase (Table 1), strongly suggest Ca^{2+}-dependent protein phosphorylation systems as potential sites of its action. It is conceivable that this inhibition is most likely to occur during ischemia because of the increased palmitoylcarnitine concentrations (19). Adams *et al.* (24) have reported that the cardiac membrane ATPase activities are sensitive to palmitoylcarnitine inhibition, with IC_{50} values of about 40–70 μM. In comparison, phosphorylation of the particulate proteins by Ca-PKase was relatively resistant to its inhibition, with IC_{50} values of about 390–500 μM (Table 2). It is possible, therefore, that binding of the acylcarnitine to the membranes can differentially affect the membrane-associated biological events. Although direct evidence is still lacking, it appears that the effects of palmitoylcarnitine on Ca^{2+}-dependent protein phosphorylation (Fig. 4 and Table 2), ATPase activities (24), and membrane properties (18–20, 31) may in part account for the pathophysiologic manifestations in the heart after myocardial infarction—i.e., decreased contractility and enhanced excitability of the myocardium.

Note Added in Proof. We have observed that phosphorylation of cardiac proteins catalyzed by phospholipid-sensitive or calmodulin-sensitive Ca-PKase was similarly inhibited by adriamycin (35), an antineoplastic drug with a unique cardiotoxicity.

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