ALTERATIONS IN PROTEIN KINASE A AND PROTEIN KINASE C ACTIVITIES AND PROTEIN LEVELS IN CARDIOMYOPATHY

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by

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ABSTRACT

Although protein kinases are known to regulate cardiac function, very little is known about their status in cardiomyopathic heart. Since protein kinase C (PKC) is crucial in a number of receptor mediated processes for protein phosphorylation, we examined changes of PKC (PKC-α, -β, -ε and -ζ isozymes) in diabetic hearts. In this study, rats were made diabetic by an intravenous injection of streptozotocin (65 mg/kg body wt) and the hearts were removed 8 weeks later. At 6 weeks, some of the diabetic animals received subcutaneous injection of insulin (3U/day) for 2 weeks. PKC activity was assayed by measuring the transfer of ^{32}P from [γ-^{32}P] ATP to a synthetic substrate. Relative protein content of the specific PKC isozymes was obtained by using immuoblot analysis in cytosolic, particulate and homogenate fractions from control, diabetic and insulin-treated diabetic rat hearts. PKC was increased by 43% in the homogenate and 90% in the cytosolic fraction from the diabetic hearts compared with control preparations as determined using an activity assay, and these values corresponded to increases of 125% to 500% of specific PKC isozymes in the cytosolic fraction of the diabetic group. No significant change in PKC was observed in the particulate fraction of the diabetic group. Furthermore, these changes were partially reversed upon administration of insulin. These results suggest that an increase in cytosolic location of PKC-α, -β, -ε and -ζ isozymes may play a role in subcellular changes during the development of diabetic cardiomyopathy.

In another series of experiments, genetic cardiomyopathic hamsters (250-300 day old; UM-X 7.1) were employed to test if the observed changes in diabetic hearts are similar to other types of cardiomyopathy. Age-matched Syrian hamsters were used as control. The PKC activity obtained by assaying the cytosolic (193.2 ± 11.2 vs 71.8 ± 4.5 pmol Pi/min/mg) and particulate (20.2 ± 0.9 vs 7.1 ± 0.9 pmol Pi/min/mg) fractions was significantly increased in the failing hearts in comparison to the control preparations. The relative content of cytosolic PKC-α and PKC-ε and of the particulate PKC-ε was significantly increased (by 24%, 29% and 30%, respectively) in the failing hearts; there was no change in PKC-β and PKC-ζ content. These results indicate that increases in PKC in genetic cardiomyopathic hearts may be due to changes in PKC-α and PKC-ε isozymes at the severe stage of congestive heart failure. Furthermore, changes in PKC isozymes seem to depend upon the type of cardiomyopathy.

In order to determine whether changes in the PKC of cardiomyopathic hearts are specific in nature, we also examined PKC in skeletal muscle as well as protein kinase A (PKA) in both cardiac and skeletal muscles. The PKC activity assay showed this enzyme was elevated in the homogenate, cytosolic and particulate fractions of cardiomyopathic skeletal muscle. Furthermore, PKA were significantly increased in comparison to the control values in both cardiac and skeletal muscles of the cardiomyopathic hamsters; the relative protein content for PKA was 45% and 50% higher respectively in cardiac and skeletal muscles relative to cardiomyopathic

hamsters. These results indicate that alterations in PKA and PKC due to cardiomyopathy are not limited to the heart. Furthermore, the changes in cardiac and skeletal muscle PKC are not specific in nature.

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I. LITERATURE REVIEW

1. Introduction

Cardiomyopathy is a primary disorder of the heart muscle that causes abnormal performance of the heart (1). This distinct cardiac problem is not the result of pericardial, hypertensive, congenital, valvular or ischemic disease but rather three basic types of functional cardiomyopathies have been described: (a) dilated cardiomyopathy which is the most common form, and generally characterized by ventricular dilatation, contractile dysfunction, and symptoms of congestive heart failure; (b) hypertrophic cardiomyopathy which is marked by inappropriate left ventricular hypertrophy with asymmetrical involvement of the interventricular septum; and (c) restrictive cardiomyopathy which is recognized by impaired diastolic filling. It should be mentioned that the distinction between these three functional categories of cardiomyopathy is not absolute and often there is overlap. The incidence of cardiomyopathies appears to be increasing; there are 5-8 more cases of dilated cardiomyopathy per 100,000 population reported annually (2).

Although mechanisms of heart dysfunction in cardiomyopathies are poorly understood, dramatic alterations of myocardial metabolism in cardiomyopathic hearts have been reported. The changes in heart function and structure in cardiomyopathy are considered to be the consequence of energy depletion, increased sympathetic activity, microangiopathy and intracellular Ca^{2+} overload (3,4). The

hypothesis concerning the involvement of increased sympathetic activity in the genesis of cardiomyopathy is of crucial importance because it can be seen to explain the observed changes in myocardial metabolism as well as defects in the signal transduction systems of cardiomyopathic hearts. Abnormalities in signal transduction are evident from the occurrence of β-adrenoceptor desensitization and uncoupling from the adenylyl cyclase, while the α-adrenoceptor density is increased in the late stages of cardiomyopathy (5,6). Abnormalities in sympathetic nervous system in human cardiomyopathy have been observed in different types of heart failure including idiopathic dilated cardiomyopathy and ischemic dilated cardiomyopathy (7-9); it should be recognized that all patients were on various cardiac drugs and thus the results are difficult to interpret in terms of pathophysiological changes in heart dysfunction. Because of the difficulties in assessing the cellular, metabolic and molecular mechanisms involved in the dysfunction of cardiomyopathic hearts in humans, several investigators have employed different experimental animal models of cardiomyopathy (10-12). For example, both rat model of diabetic cardiomyopathy and cardiomyopathic hamster model of genetic cardiomyopathy have been extensively used for studying the subcellular basis of heart dysfunction (13-15). It is therefore proposed to deal with these experimental models for obtaining an in-depth analysis of the existing information regarding the pathophysiology of heart dysfunction in cardiomyopathy. In view of the lack of information on the signal transduction mechanisms involving

protein kinases, which are known to regulate various subcellular and metabolic processes, it is intended to examine the evidence on how changes in some protein kinases may be associated with heart dysfunction in cardiomyopathy.

2. Experimental models of cardiomyopathy

A. Diabetic cardiomyopathy

Diabetes mellitus is marked clinically by the presence of abnormalities in carbohydrate metabolism (10). It is associated with an excessive cardiovascular morbidity and mortality. In fact, diabetes has been reported to be the third leading cause of death in USA behind cardiovascular disease and cancer (16). Generally there are two major types of diabetes mellitus: insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM) (17). Although cardiac dysfunction is often associated with coronary atherosclerosis in diabetic patients, evidence has accumulated for the presence of diabetic cardiomyopathy in humans (18). The existence of a specific cardiomyopathy in diabetes was first suggested in 1972 by Rubler et al. (19), who described findings of cardiomegaly and cardiac failure of unknown cause at autoposy in 4 out of 27 diabetic patients. Later, the term "diabetic cardiomyopathy" was introduced in 1974 by Hamby et al. (20). The Framingham Study revealed that diabetes was another discrete cause of

congestive heart failure and that some form of cardiomyopathy was associated with diabetes as a result of either small vessel disease or metabolic disorder (7).

Diabetic cardiomyopathy plays a dominant role in the genesis of cardiac dysfunction (21). Several factors including microangiopathy, alterations in myocardial metabolism, hormone imbalance and autonomic neuropathy are considered to explain diabetic cardiomyopathy. The structural effects of diabetes on rat heart included focal loss of myofibrils and swelling of transverse tubules as well as sarcoplasmic reticulum (SR) (22). All these cytoplasmic alterations were accompanied by intercellular and perivascular deposition of connective tissue in the heart and were partially reversible by treatment of diabetic animals with insulin. Previous studies in our laboratory have shown alterations in cardiac myofibrils, SR and sarcolemma (SL) during the development of diabetes (23-26). Experimental diabetes in many animal species is usually induced by the use of streptozotocin or alloxan. Streptozotocin has gained a widespread use as diabetogenic agents as it is selectively toxic for pancreatic β-cells; the streptozotocin-induced diabetes bears a close resemblance to IDDM in humans (27).

B. Genetic cardiomyopathy

UM-X7.1 line of cardiomyopathic hamsters has been established as a model for genetic cardiomyopathy by cross-breeding homozygous diseased animals of the BIO 14.6 strain with unrelated healthy hamsters and by recovering the mutant gene

in the F2 generation (28). This hamster model provides unique possibilities for studying the frequent involvement of the myocardium in human primary muscle disorders (29). The polymyopathy is transmitted by an autosomal recessive gene. The resulting lines will have a disease incidence of 100% with no significant difference in the histologic features of muscle lesions, indicating that the genotype of this hereditary disorder is constant and reliable. The average life-span of these cardiomyopathic hamsters is about 300 days, which is almost one-third of the normal life-expectance of this species. They showed more severe cardiac lesions with 100% incidence of congestive heart failure compared with the BIO 14.6 strain of hamsters (30). However, the time course and severity of the disease appear to vary somewhat between different lines and even between sibling of the same line (28).

Myocardial necrosis in UM-X7.1 strain of cardiomyopathic hamsters begins to appear at 30-40 days and reaches a maximum at 60-75 days of age. This necrotizing phase is followed by cardiac hypertrophy at 90-120 days, and thereafter varying degrees of congestive heart failure (3). The earliest necrotic changes under the microscope consist of focal myolysis in the absence of cellular infiltration. In contrast to the skeletal muscle, the pathologic changes in the heart give rise to substantial scarring. Cardiomyopathic hamsters (UM-X7.1) show clinical signs of congestive heart failure and an abnormal EKG pattern (30). The gross signs of circulatory failure consist of liver enlargement and fluid accumulation together with

an appreciable increase of heart volume (28). On the basis of general characteristics, cardiomyopathic hamsters at the age of 120-160 days old are at the early stage of congestive heart failure, while those at 160-200, and 200-300 days old are considered at being at moderate and severe stages of heart failure (3). Most of the animals typically die from cardiac failure before they are about 300 days old. Several sites of cellular dysfunction have been identified in the cardiomyopathic myocardium. These include defects in mitochondria (31), sarcoplasmic reticulum (11), myofibrils (32) and sarcolemma (33).

A recent study has shown a dramatic effect of reduced skeletal muscle bulk on the ventilatory response to exercise in chronic congestive heart failure secondary to idiopathic dilated and ischemic cardiomyopathy (34). Abnormalities in contractile proteins have also been identified in the sarcomere of skeletal as well as cardiac muscle of some but not all subjects with familial hypertrophic cardiomyopathy (35). In UM-X 7.1 polymyopathic hamsters, not only the cardiac muscle but the skeletal muscle is also involved (36). There are scattered white streaks in skeletal muscle at autopsy; however with the progression of cardiomyopathy, the lesions become more confluent involving bundles of fibers. At an early stage, the light microscopic changes involve hyalinization and fragmentation of isolated myofibers with little cellular infiltration. Isolated groups of myofibers undergo myolysis in the absence of cellular reaction but more often the degenerative changes consist of coagulation

necrosis with phagocytosis, which includes small regenerative myofibers showing increased basophilia and internal proliferation of nuclei (37,38).

3. General functions of protein kinases

The eucaryotic cell is a highly regulated entity capable of responding to its immediate intracellular environment as well as to external stimuli (39). Transmembrane signaling by extracellular agonists is generally mediated by specific receptors and often results in alterations of the intracellular concentrations of either cyclic AMP (cAMP), calcium, inositol polyphosphates or diacylglycerol (DAG). These second messengers affect many physiological processes through mechanisms which often involve covalent phosphorylation of appropriate rate-limiting enzymes, ion channels or other proteins (40-42). Protein kinases are also involved in signal transduction. These enzymes phosphorylate specific proteins essential for the activation of a biological process (43,44). When these proteins are phosphorylated by protein kinases in the cell, their activities are either increased or suppressed (45,46). In the heart, protein kinases are involved in the regulation of cation transport, contractility, metabolism, gene expression and cellular growth. The majority of protein kinases transfer the γ-phosphate group from ATP to hydroxyl groups of Ser/Thr residues (protein Ser/Thr kinases) or Tyr residues (protein Tyr kinases) in proteins and thus induce a change in the activity or function of the

substrate enzyme or protein (47,48). In some cases, a single protein kinase step lies between receptor activation and biological response whereas in others, a series of protein kinases participates in a linear or branching cascade that allows signal amplification and integration (47). Therefore, it is logical to conclude that in seeking biological roles for protein kinases, demonstration of a change in the activity/function of the particular protein kinases is of paramount importance. Two multifunctional protein kinases, namely cyclic AMP-dependent protein kinase (protein kinase A) and calcium/phospholipid-dependent protein kinase (protein kinase C), are thought to mediate many of these phosphorylation reactions (49). Accordingly, the functional implications of these protein kinases will be discussed with respect to cardiac cell function in health and disease.

A. Role of protein kinase C (PKC) in heart function

(i) Structure and function of PKC

PKC was first discovered in 1977 but at that time it had no obvious role in signal transduction (50). Later, it was shown to be a Ca^{2+}-activated, phospholipid-dependent enzyme, which was firmly linked to signal transduction upon demonstration that DAG greatly increase the affinity of PKC for Ca^{2+}, thereby activating it (51). Molecular cloning studies indicated that PKC was not a single entity but rather comprised a family of at least 12 distinct isoforms (52,53). All PKC

isoforms contain a highly conserved carboxyl terminal kinase domain that includes an ATP-binding site. PKC isoforms differ, however, in their amino-terminal regulatory regions. The conventional PKC isoforms (cPKC: α, β, and γ) contain a calcium-binding domain which accounts for their sensitivity to activation by calcium. A second regulatory domain, consisting of two adjacent cysteine-rich, zinc finger-like motifs, is also present in the amino-terminal portion of the molecule and is believed to be responsible for diacylglycerol/phorbol ester binding. On the other hand, the novel PKC isoforms (nPKC: δ, ε, η/L, and θ) differ structurally from the conventional isoforms in that they lack the putative calcium-binding domain and, as a result, do not require calcium for maximal enzymatic activation. Atypical PKC isoforms (aPKC: ζ, λ, ι, and μ) are distinguished from other members of the PKC gene family by the presence of only a single copy. Although PKC-μ is an additional atypical PKC, it has two cysteine-rich motifs which are unusually spaced (54-58).

Various isoforms of PKC are present in the heart; however, the concentrations of most isoforms are much less than those in the brain (59). By using an immunoblotting approach, cardiac tissue has been shown to contain two cPKC isoforms, namely PKC-α and PKC-β. In addition, the expression of PKC-ε and PKC-η, PKC-ζ, PKC-δ and PKC-θ have also been reported in the ventricular myocardium (60-63). It is premature at this time to state whether any of the newly identified PKC isoforms are present in the heart; nPKC-θ immunoreactivity was

recently reported to be undetectable. The pattern of PKC expression defined by Northern blotting agreed largely with that defined by immunoblotting (64).

PKC isoforms are physiologically activated by neurohormones which bind to G-protein-coupled receptors (65,66). Activation of these receptors, including the α_1-adrenergic, the P_2-purinergic, the endothelin, the m_1-muscarinic and angiotensin II receptors, most likely activate the Gq-coupled phospholipase C_β (PLC-β)(67-71). This results in breakdown of phosphatidylinositol bisphosphate (PIP_2) generating both inositol triphosphate (IP_3) and (DAG). IP_3 has been shown to stimulate Ca^{2+} release from the sarcoplasmic reticulum and thus increase the intracellular concentration of Ca^{2+}, whereas DAG is a powerful activator of most PKC isoforms (62). In addition, phospholipase D (PLD) is considered to be activated via G protein by a variety of stimuli, including some growth factors. PLD hydrolyzes phosphatidylcholine (PC) with the formation of phosphatidic acid (PA), then producing DAG through the action of phosphatidate phosphohydrolase (PP), which complements the formation of DAG through the PLC pathway (Figure 1) (72). The time course of DAG is usually biphasic due to its initial formation from PIP_2 via PLC-β and another pathway from PC through PLD and PP. It is already accepted that the early phase of DAG production is derived from the hydrolysis of PIP_2 by PLC-β and the second late phase is from PA, which is produced by PLD (73).

Activation of PKC may influence the activity of a variety of cation channels with inotropic consequences. Mg^{2+}-, K^+-, Na^+- and Ca^{2+}-channels have been

reported to be modulated by PKC (74-81); however, the results are still controversial. The first well identified cardiac-specific PKC substrate is a membrane bound 15 kDa protein, phospholemman (82). Although it has been suggested that phospholemman could be a chloride channel, definitive proof regarding the physiological significance of its phosphorylation is yet not available (83). It was shown that the slow inward (L-type) Ca^{2+}-current is increased by phorbol 12-myristate 13-acetate (PMA) which increases the intracellular Ca^{2+} concentration (84). It has also been suggested that PKC-induced modification of the Na^{+}/H^{+} exchanger is responsible for cellular alkalization (85-87) and is thus involved in the inotropic effect because the myofibrillar sensitivity to Ca^{2+} is increased. The sarcoplasmic reticular protein, phospholamban, is a good in vitro substrate for PKC. The PKC-induced phosphorylation of phospholamban increases the SR Ca^{2+}-stimulated ATPase activity which in turn stimulates Ca^{2+}-uptake from the cytosol and thus promotes relaxation of cardiac muscle (88).

Contractile proteins are among the best substrates for cardiac PKC. Both the thick-filament proteins (C-protein and myosin light chain 2 (MLC2)) and the thin filament proteins (the troponins TnI and TnT) are targets for endogenous PKC (89,90). Phosphorylation of TnI and TnT by PKC in reconstituted actomyosin resulted in a decrease of Ca^{2+}-stimulated Mg^{2+}-ATPase activity and this was reversed by dephosphorylation (91). PKC can also phosphorylate C proteins but the significance of the phosphorylated C proteins is not clear at present (92,93). It is

reported that PKC plays a role in myosin isoform shift, which is related to the decreased myofibrillar ATPase activity (91,94). PKC-mediated phosphorylations of TnI and C-protein gives rise to different functional consequences from that by PKA-mediated phosphorylation. Whereas phosphorylation by PKA decreases the sensitivity of myofibrils to Ca^{2+}, phosphorylation by PKC decreases the maximal activity of the Ca^{2+}-activated actomyosin ATPase and thus contributes to the negative inotropic effects (93). Phosphorylation of MLC2 by MLC kinase has also been shown to result in an increase in Ca^{2+} sensitivity and likewise the Ca^{2+} sensitivity is also increased by PKC-mediated phosphorylation (Figure 1) (95). Since the effects of PKC activation are complex and alter with exposure time, the resultant influence of PKC on the cardiac contractile state is difficult to assess. Whether phosphorylation through PKC is related to potentially positively or negatively inotropic influences seems to depend upon the species employed for experiments.

Although activation of PKC can initiate a cascade of regulatory events, which include both positive and negative interactions, PKC appears to play an important role in the phosphorylation of regulatory proteins and modulation of gene expression in the myocardium. One of the consequences of PKC activation is the activation of transcription for increasing protein synthesis in the myocardium (96). This is at least partly mediated by the binding of the activator protein-1 (AP-1) complex to a consensus sequence present in the promoter regions of many genes. AP-1 was

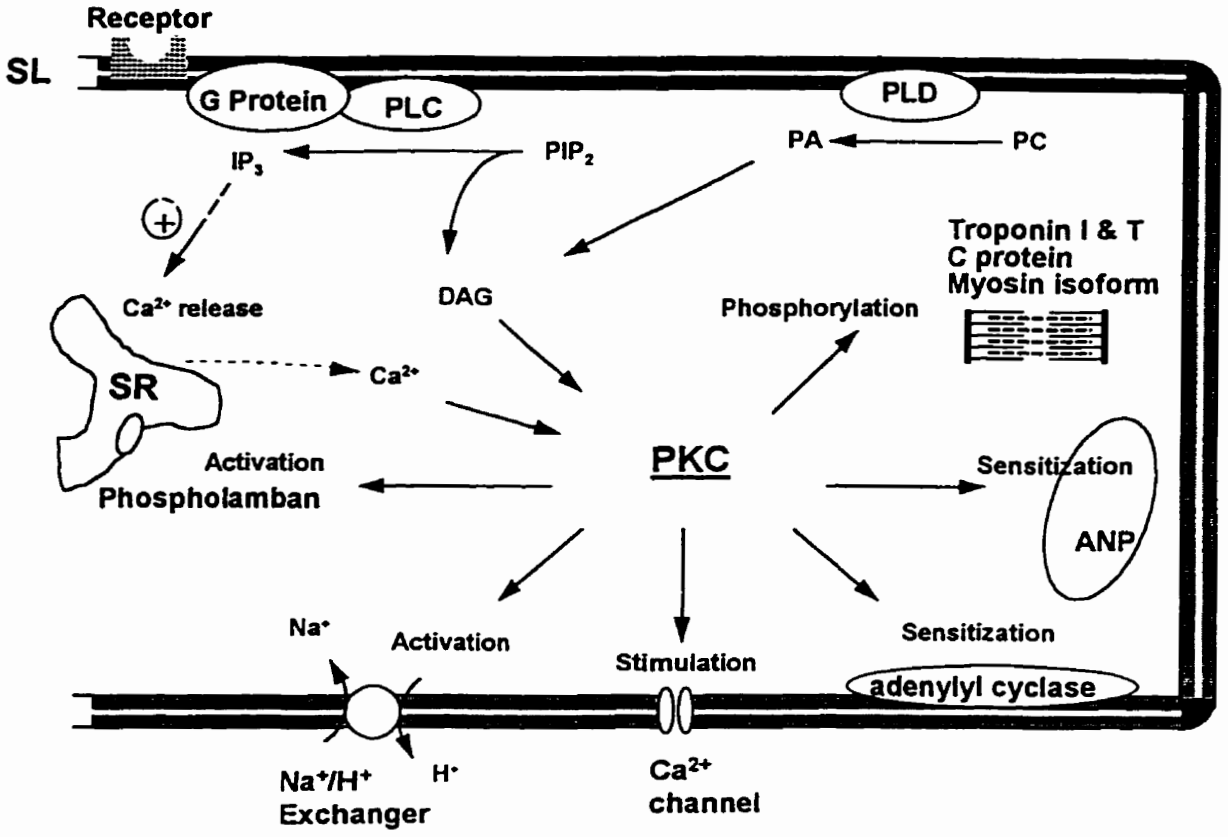

Figure 1 General scheme depicting PKC functions.

SL: sarcolemma; PLC: phospholipase C; PLD: phospholipase D; PC: phosphatidylcholine; PA: phosphatidic acid; PIP_2: phosphatidylinositol bisphosphate; IP_3: inositol triphosphate; DAG: diacylglycerol; PKC: protein kinase C; SR: sarcoplasmic reticulum; ANP: atrial natriuretic peptide.

subsequently identified as a heterodimer of the c-jun and c-fos transcription factors. Activation of PKC could also promote the phosphorylation of Ser residues in the N-terminal DNA-binding region in c-jun by activation of mitogen activated protein (MAP) kinases pp54 and pp42/44 (97-99).

(ii) Alterations of PKC in diseased heart

(a) Cardiac hypertrophy

There are two pathways available for tissue growth: hyperplasia (increased cell division) and hypertrophy (cell growth in the absence of cell division) (100). The mammalian ventricular myocyte is a terminally differentiated cell which loses its potential for mitotic division soon after birth. Because the cardiac cell loses its ability to divide, increase in cell size is the only way for the heart to adapt to and to face an increase in hemodynamic load and thus to increased contractile work (101). In the whole heart, cardiac hypertrophy usually results from a requirement for increased pressure-volume work (102). On a more local level, the ventricular myocytes, which survive following myocardial infarction, are also able to increase their size to replace the lost of myocytes (103). This process consists of an increase in protein synthesis and an alteration of muscle phenotype but is not accompanied by DNA synthesis or cell division (48). The etiology of hypertrophy is likely to be multifactorial and a large number of agonists may be involved during different stages of its development.

In cultured ventricular myocytes, a variety of agonists have been found to stimulate hypertrophy. These include agents such as endothelin-1, α_1-adrenergic agonists, and angiotensin II, which are coupled to PLC-β for increasing the formation of DAG and activation of PKC (48,104-114). Activation of PKC, in turn, appears to be one of the events that can initiate a hypertrophic response in cardiomyocytes through the activation of MAP kinase (115,116). In addition, the endothelin-1 induced development of hypertrophy via activation of distinct PKC isoenzymes may be initiated not only by PLC but also by PLD signaling pathway (117). It should be noted that the hypertrophic process is associated with a genetic program which consists of the expression of immediate early genes (c-*myc*, c-*fos*, c-*jun* and *Egr1*) within 30-60 minutes followed by an expression of fetal genes, such as skeletal α-actin, β-myosin heavy gene and atrial natriuretic peptide (ANP) (100,118,119). Finally, hypertrophy is accompanied by an overexpression of constitutively expressed MLC2 and cardiac α-actin genes and by an assembly of contractile proteins into sarcomeric units. PKC has been shown to play a role in this genetic program (120). It is pointed out that PKC does not directly couple the neurohormonal receptor with gene expression, but rather it belongs to a cascade of kinases including Raf, tyrosine kinase, and MAP kinase which are also activated during the hypertrophic process (98,120-122).

By increasing myofibrillar protein content and sarcomere assembly in individual myocytes, myocardial hypertrophy provides an adaptive response to

hormonal and mechanical stimuli which increase demand for contractile work. Virtually every phenotypic feature of the hypertrophic response has been shown to be induced by chronic stimulation with phorbol esters. These include an increase an increase in the rate of transcription of rDNA, which leads to an increase RNA content and an increase in the capacity for protein synthesis (100). These changes are followed by rapid and transient induction of immediate early gene expression, transcriptional activation of several fetal genes, accumulation of contractile proteins, assembly of contractile proteins into organized sarcomeric units and an increase in cell size (123). It has been suggested that both PKC-α and PKC-β act simultaneously to induce the central members of the immediate early gene program expression, c-*fos* and c-*jun*, which heterodimerize and bind the consensus AP-1 sequence in the promoter region of phorbol ester-inducible genes thereby exerting regulatory effects on gene transcription (115,120). Kariya et al. (124) have demonstrated that β-myosin heavy chain is activated preferentially by PKC-β. The studies implicate PKC in responses leading to myocardial hypertrophy upon transaction of early immediate genes and contractile protein genes for an enhanced protein synthesis following hormonal or mechanical stimuli (125).

(b) Ischemic preconditioning

Transient periods of myocardial ischemia of insufficient severity to cause cell death lead to reversible dysfunction, a phenomenon often referred to as myocardial "stunning" (126). On the other hand, ischemic preconditioning means that exposure

of the heart to a short ischemic episode results in protection against a longer period of ischemic insult. Unfortunately, this protection is lost within 1-2 hr (127). There is much current interest in the potential role of PKC in ischemic preconditioning (128) because short-term adaptation induced by ischemic preconditioning includes attenuation of myocardial dysfunction and reduction of cell necrosis as well as life-threatening arrhythmias (129). Protection is believed to be mediated by specific cell signaling mechanisms, suggesting that different parts of the signal transduction cascade might represent potential targets for the pharmacological induction of ischemic preconditioning. There is now evidence that PKC could play a major role in the preconditioning phenomenon (130,131). Calphostin (132), staurosporine (133) and polymyxin B (134), three PKC inhibitors, were found to prevent preconditioning in rabbit isolated myocytes or whole heart. Disruption by colchicine of cytoskeletal microtubules which are involved in PKC translocation from the cytosol to the membrane was also shown to block the protective effect of preconditioning against cardiac infarct. Activation of endogenous PKC by PMA was shown to mimic preconditioning in rabbit heart (135-137). It has been suggested that adenosine released during brief ischemia activates adenosine A_1 receptors which, through a pertussis toxin-sensitive G-protein, stimulates PLC and results in the formation of DAG and activation as well as translocation of PKC from the cytosol to the myocyte membranes. PKC has also been considered to phosphorylate an unidentified membrane-bound effector protein during the early minutes of subsequent sustained

ischemia, and thereby elicit the well-described reduction of infarct size (138-140). The delay of infarct size development by ischemic preconditioning involves the activation of PKC in rats and rabbits. In dogs, the role of PKC in ischemic preconditioning is controversial and no prevention of ischemic preconditioning by PKC inhibitor occurred in swine (141).

Various investigators have concluded that changes in SR Ca^{2+} during the preischemic phase are responsible for cardioprotection by preconditioning of the ischemia-reperfusion induced alterations and that this functional protection is PKC regulated via the translocation of specific isoforms (142,143). It was also indicated that Ca^{2+} administration during the preischemic phase improved myocardial functional recovery; this protective effect of Ca^{2+} was abolished by PKC inhibition (124,144). More recently, a second delayed period of protection has been demonstrated several hours after the preconditioning stimulus; other stimuli such as heat shock, bacterial lipopolysaccharide, interleukin 1, tumor necrosis factor and catecholamines have also been suggested to lead to delayed preconditioning (145). Delayed preconditioning is associated with induction of proteins including heat shock proteins and antioxidant enzymes, which are cardioprotective. Although the mechanisms by which induction of these cardioprotective proteins occurs is not clear at present, the potential role of PKC in induction of cardioprotective genes merits further investigation.

(c) Cardiomyopathy

Not much information is available in the literature regarding changes in protein kinases in different types of cardiomyopathic hearts. Some investigators have shown that cardiac PKC activities were significantly increased in 180 day-old cardiomyopathic hamsters and PKC potentiated the cAMP-dependent phosphodiesterase (PDE) activity in hypertrophic cardiomyopathy (146,147). In diabetic rat cardiomyopathy, it has been reported that cardiac PKC activity is elevated but the changes of different PKC isoforms have not been explored (148,149). Increased cardiac PKC activity may cause excessive accumulation of intracellular Ca^{2+} in the myocardium and this may play an important role in the pathogenesis of cardiac dysfunction in diabetic cardiomyopathy (15).

B. Role of protein kinase A (PKA) in heart functions

(i) Structure and function of PKA

Over the past 20 years, many laboratories have focused their investigation on the β-adrenergic regulation of cardiac contractility through phosphorylation of cellular substrates by PKA. Based on these studies, there is now general agreement that PKA-mediated protein phosphorylation has an important role in heart function. Stimulation of cardiac myocytes by catecholamine and other β-adrenergic agonists produces an elevation of the intracellular concentration of Ca^{2+}; this occurs primarily through PKA-mediated phosphorylations at sarcolemmal Ca^{2+} channels and the SR

Ca^{2+} pump regulatory protein, phospholamban (84,150-152). The resulting increased availability of Ca^{2+} to the contractile apparatus causes an increase in the force of cardiac contraction. Direct phosphorylation of the contractile machinery by the activation of PKA itself is also recognized as having an important modulatory role in cardiac contraction-relaxation cycle. It is now well established that β-adrenergic stimulation of either intact hearts or isolated myocardial cells results in the PKA-mediated phosphorylation of C-protein and troponin I in the thick and thin myofilaments, respectively (153).

The participation of the cyclic AMP-dependent PKA in the mediation of β-adrenergic effects on glycogen metabolism was the first biological event ascribed to Ser/Thr protein kinases (45,154). Glycogen metabolism is less biologically important in the heart than in skeletal muscle because the carbohydrate fuel required by the heart is largely supplied exogenously under normal conditions. Nonetheless, the intracellular glycogen reserves are significant under conditions of hypoxia or ischemia where these are rapidly depleted (155). At any rate, PKA plays an important role in mediating β-adrenergic effects on the contractile activity of the cardiac myocyte and the importance of the β-adrenoceptor in the regulation of cardiac contractility has been emphasized by the finding that cardiospecific overexpression of the β_2-adrenoceptor transgene increases ventricular function in vivo (156). PKA is also known to modulate cardiac myocyte gene expression by its phosphorylation of the cAMP-responsive element binding protein (CREB-P)

transcription factor (157). These studies have emphasized the role of PKA in heart function, metabolism and cellular growth.

In its holoenzyme state, PKA is an inactive tetramer composed of a regulatory (R) subunit dimer and two catalytic (C) subunits. Each of the PKA two subunits consists of a regulatory subunit, which binds two molecules of cAMP and a catalytic subunit. Combination with cAMP causes the R_2C_2 complex to dissociate and release the active C monomers (158). When cAMP is elevated in the heart by β-adrenergic stimulation, the holoenzyme is activated. Four molecules of cAMP bind the R subunit dimer carrying the release of the C subunits which phosphorylate a wide variety of substrates such as cation channels, contractile proteins, and metabolic enzymes (Figure 2) (151,159). The two major classes of holoenzyme, PKA I and PKA II, are present in the heart but their relative abundance is species-dependent; PKA II is by far the most abundant isozyme in the bovine heart, whereas PKA I is the predominant isozyme in the rat heart (160). It should be pointed out that β-adrenergic agonists are positively inotropic, lusitropic and chronotropic in the heart. Positive inotropy is mediated by increased rates of Ca^{2+} entry into the cytoplasm of the cardiac myocyte whereas positive lusitropy is modulated by enhanced uptake of Ca^{2+} into the SR as well as by increased rates of release of Ca^{2+} bound to myofibrillar troponin C; these reactions involve PKA (161,162).

PKA is now known to regulate a variety of substrates, such as L-type Ca^{2+} channels, phospholamban as well as the troponin I and T subunits (163,164). The

role of β-adrenoceptor regulation of the L-type calcium current is well defined where receptor binding stimulates the GTP-binding protein, G_s, increases the production of cAMP for the activation of PKA and the enhancement of Ca^{2+} currents (165). Although the molecular basis for the regulation of cardiac L-type Ca^{2+} channel activity by PKA remains unclear, direct PKA phosphorylation of the bovine ventricular α_1 subunit of Ca^{2+} channels in vitro has been demonstrated. It was shown that the α_1 subunit of the cardiac L-type Ca^{2+} channel is sufficient for substantial modulation of Ca^{2+} channel activity by PKA and for potentiation by rate-dependent protein phosphorylation. Voltage-dependent potentiation of the activity of the α_1 subunit may contribute to the increase in contractile force in response to increased rate of stimulation, the positive staircase effect in heart muscle. Functional and biochemical studies suggest that the direct phosphorylation of the α_1 subunit is involved in modulation of cardiac and skeletal muscle channel function by PKA (166). Efforts have also been made to identify the amino acid residues phosphorylated by PKA in vitro. Full length L-type calcium channel α_1 subunit are rapidly phosphorylated by PKA in vitro and in vivo at sites located in their long carboxyl terminal tails (166-168). Ser^{1627} and Ser^{1700} may represent sites of PKA phosphorylation involved in the physiological regulation of cardiac L-type Ca^{2+} channel function (169). Calcium ions entering cells through the L-type Ca^{2+} channels are important regulators of a variety of cellular processes and initiate Ca^{2+}-induced Ca^{2+} release and contraction of cardiac muscle. For myofibril proteins,

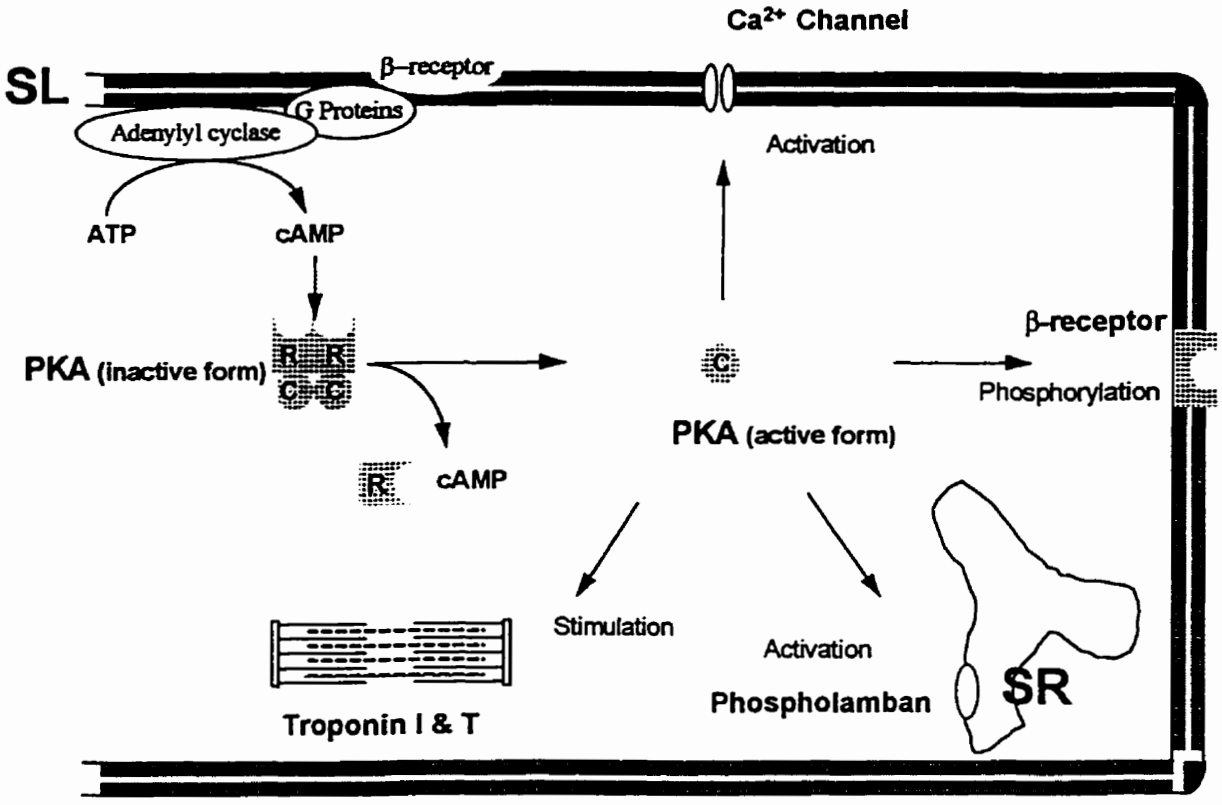

Figure 2 General scheme depicting PKA functions.

SL: sarcolemma; SR: sarcoplamic reticulum; ATP: adenosine triphosphate; cAMP: cyclic adenosine monophosphate; PKA: protein kinase A; R: regulatory subunit; C: catalytic subunit.

troponin I and T are the substrates of PKA. Two serine residues in amino terminus of troponin I subunit are believed to be phosphorylated by PKA upon stimulation by β-agonists resulting in decreased sensitivity of myofibrils to Ca^{2+} by increasing release of Ca^{2+} from the troponin C/Ca^{2+} complex (170). It has been shown that increased dissociation of Ca^{2+} from troponin C coupled with the faster uptake of Ca^{2+} by the SR following PKA-dependent phosphorylation of phospholamban can account for the faster relaxation seen in the inotropic response of the heart to catecholamines (Figure 2)(170,171).

(ii) Alterations of β-adrenergic receptor system in congestive heart failure

Although the sympathetic drive is increased in heart failure, the cells of the failing heart become desensitized to the increased level of β-adrenergic receptor agonists (153,172). The β-adrenergic receptor density is decreased in response to exposure to isoproterenol, which is accompanied by decreased stimulation of adenylyl cyclase by the agonist. These effects of high doses of isoproterenol are thought to mimic the in vivo situation in heart failure, where the sympathetic stimulation is elevated to maintain the ventricular function. There is some evidence that the levels of G_s are reduced whereas those of G_i are increased in the cardiomyopathic hearts (173). All these changes may contribute to the reducing contractility and ability of the failing heart to respond to exercise. Translocation of the receptor to intracellular membranes and phosphorylation of the receptor by the

β-adrenergic receptor kinase as well as PKA are involved in the desensitization and uncoupling of the receptor from the G protein (118,173,174). However, alterations in PKA during the development of heart failure remain to be investigated for a full appreciation of defects in the signal transduction mechanisms in the failing heart.

II. STATEMENT OF THE PROBLEM AND OBJECTIVES

Although cardiomyopathy is known to result in cardiac dysfunction and heart failure, the precise subcellular mechanisms of the pathophysiological alterations in cardiomyopathy are not very clear. The work from several laboratories has indicated there is a depression in SL function and down regulation of β-adrenergic receptors in cardiomyopathy whereas the α-adrenoceptor density was found to be increased in the severe stage of congestive heart failure due to cardiomyopathy. In addition, the investigations in experimental animals and clinical patients have suggested that the presence of serious defect in the contractile function of the diseased heart is associated with decreased myofibrillar ATPase, actomyosin ATPase activities, myosin Ca^{2+} ATPase activity, myosin isozyme shift and myofibrillar phosphorylation in cardiomyopathy. Since PKC and PKA are the central factors in connecting the environmental stimuli to the biological effects, as well as for modulating the gene expression of myosin heavy chain β-isozyme and phosphorylation of myofibrils, it is logical to assume that these protein kinases may become defective during the development of cardiomyopathy. Because very little information regarding the function of protein kinases in different kinds of cardiomyopathies is available in literature, the present study was undertaken to investigate changes of protein kinase activity and their protein contents in diabetic and genetic cardiomyopathies. Furthermore, this study also examines whether the

protein kinase alterations in diabetic cardiomyopathy are reversible by in vivo insulin administration. In addition, since both the cardiac and skeletal muscles are defective in the UM-X 7.1 cardiomyopathic hamsters, changes of protein kinases in skeletal muscle were carried out in this study as well. It is hoped that this investigation will provide comprehensive information regarding the pathogenesis and pathophysiology of cardiomyopathy and may be useful for developing a clinical therapeutic strategy by using agents which interact with protein kinases for correcting subcellular defects in cardiomyopathic patients.

III. MATERIALS AND METHODS

1. Experimental models

A. Diabetic cardiomyopathy in rat

Male Sprague-Dawley rats weighing approximately 175-200 g were randomly separated into control and experimental groups. The experimental animals were injected intravenously of 0.1 M citrate-buffered streptozotocin (pH 4.5) at a dosage of 65 mg/kg body wt. Control animals received a similar injection of the vehicle alone. Some randomly selected diabetic animals at 6 weeks after the streptozotocin injection received subcutaneous injection of 3 U protamine zinc insulin/day for 2 weeks. All animals were maintained for 8 weeks on normal rat chow and water *ad libitum* and then killed by decapitation. After removing the heart, the atria and large vessels were carefully trimmed, the ventricles were weighed and frozen at -72° C for further use. Blood samples were analyzed for glucose and insulin levels by using the Worthington Statzyme Reagent Kit and standard radioimmunoassay techniques (Amersham), respectively. The experimental model employed in this study is similar to that used previously in our laboratory (24,25,175-178).

B. Genetic cardiomyopathy in hamster

250-300 Day-old genetic cardiomyopathic (UM-X7.1) male hamsters in late

stage of congestive heart failure and healthy age-matched male control Syrian hamsters were employed in this study. The hamsters were maintained under controlled housing conditions with free access to laboratory chow and tap water. After weighing, the hamsters were decapitated, the heart, the skeletal muscle from quadriceps in the rear thigh, and the liver were removed quickly. The heart was perfused with cold Krebs-Ringer phosphate buffer containing 5.5 mM glucose to remove blood and weighed. The ventricles and skeletal muscles were put into liquid nitrogen and stored at -72°C for further use.

2. Preparations of tissue extract for PKC determination

The preparation of tissue exact for PKC was carried out by the method described by other investigators (148,179,180); all procedures were done at 4°C. The ventricular tissue (about 1 g) was minced in 4 ml of buffer (50 mM Tris-HCl, 0.25 M sucrose, 10 mM EGTA, 4 mM EDTA, 20 μg/ml leupeptin, 200 U/ml aprotinin, pH 7.5), homogenized in a Polytron (Brinkmann PT3000) at a setting of 8 for 2 × 30 sec and sonicated for 2 × 15 sec. The homogenate was centrifuged at 100,000 × g for 60 min in a Beckman ultracentrifuge (Beckman L70). The resulting supernatant was saved as cytosolic fraction and put on ice until use for running through column. The pellet was resuspended in 2 ml of buffer with 1% Triton X-100. After incubation on ice for 60 min, the resuspended pellet was centrifuged at

100,000 × g for 60 min; this supernatant was used as particulate fraction. In some experiments, the homogenate was also incubated with 1% Triton X-100 for 60 min on ice and the supernatant was prepared as described for the pellet and was used as homogenate fraction. The homogenate, cytosolic and particulate fractions were then to run through diethylamino ethyl (DEAE) cellulose columns, which were prewashed with 50 mM Tris-HCl, 10 mM EGTA, 5 mM EDTA, 0.3% w/v 2-mercaptoethanol and 200 U/ml aprotinin (pH 7.5). The enzyme was eluted from the columns with 1 ml of buffer containing 200 mM NaCl. The extraction of PKC from the skeletal muscle was carried out in a similar manner for the ventricular tissue from hamster, except 50 mg of the tissue was extracted with 1 ml of the buffer without running through the columns. Protein concentration was measured according to Lowry's method.

3. Preparation of tissue extract for PKA determination

About 50 mg of frozen myocardial and skeletal muscle tissues were homogenized respectively in 1 ml of a homogenizing buffer at pH 7.4 containing 5 mM histidine-HCl, 0.1 mM phenylmethylsulphonyl fluoride, 50 mM KH_2PO_4, 25 mM NaF, 10 mM EDTA, 750 mM KCl and 0.2 mM DTT. After gently mixing, the homogenate was centrifuged at 100,000 × g for 60 min at 4 °C and the supernatants were used for analysis of PKA activity.

4. Assay for PKC activity

PKC activity was determined with a PKC assay kit (Upstate Biotechnology Incorporated). Each assay tube included substrate (10 μl), inhibitor (10 μl), lipid activator (10 μl), enzyme preparation (10 μl). The reaction was initiated by the addition of [γ-^{32}P]ATP (10 μl; 1 part of [γ-^{32}P]ATP in 9 parts of kit ATP solution) and allowed to proceed at 30°C for 10 min. The reaction mixture (25 μl) was put onto the P81 phosphocellulose paper for 30 sec, which was then washed 3 times (5 min per time) with 0.75% phosphoric acid. This paper was further washed with acetone for one time and then put into scintillation vial and the bound radioactivity on the paper was counted in a scintillation counter for measuring the incorporation of ^{32}P from [γ-^{32}P] into a synthesized substrate (148,179,181,182). Preliminary experiments revealed that the presence of phosphatase inhibitor was required for the measurement of PKC activity in hamster tissues. Accordingly, okadaic acid (0.2 μM in reaction), a protein phosphatase inhibitor, was present in the reaction solution in small samples of non-purified PKC fractions from hamster samples (183). Okadaic acid is a highly specific inhibitor of type 1 and type 2A phosphatase and characterizes the nature of the endogenous PKC inhibitory activity causing a general stimulation of protein phosphorylation without affecting any of the relevant protein kinases (184). It has already shown that the functional PKC inhibitory activity was completely eliminated by okadaic acid, making it possible to measure PKC activity in non-purified cell fractions (184).

5. Assay of PKA activity

PKA activity was evaluated by using the PKA assay kit (Upstate Biotechnology Incorporated). Each assay tube contained substrate (10 μl), inhibitor (10 μl), 10 μl of extracted PKA solution. The reaction was initiated by adding [γ-^{32}P]ATP (10 μl; 1 part of [γ-^{32}P]ATP in 9 parts of kit ATP solution). The PKA activity was assayed in a final volume of 40 μl. After an incubation for 10 min at 30°C, 25 μl aliquots were put onto the P81 phosphocellulose paper for 30 sec. These phosphocellulose papers were immediately washed in 0.75% phosphoric acid for 3 times (5 min per time) and finally with acetone before putting into scintillation vials. The bound radioactivity on the papers were counted in a scintillation counter by measuring the incorporation of ^{32}P from [γ-^{32}P] into the substrates (102,151).

6. Analysis of PKC isozyme protein content

The relative protein content of PKC-α, -β, -ε, and -ζ isozymes (PKC-θ, and -δ were also investigated in hamster model) was obtained by running 10% mini sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels followed by Western blotting analysis with cytosolic and particulate fractions. The concentration of protein in these fractions was adjusted to 1 mg/ml with the homogenizing buffer and the SDS-PAGE loading buffer (1 part) was added into the homogenizing buffer (3 parts). The SDS-PAGE loading buffer contained 0.25 M

Tris-HCl (pH 6.8), 8% (w/v) sodium dodecyl sulfate (SDS), 45% glycerol, 20% β-mercaptoethnol, and 0.006% bromophenol blue. The protein loads for each group were the same (10 μl in each well). The electrophoresis was carried out at 200 volts for 40-45 min. The proteins in both fractions separated by SDS-PAGE were electroblotted to Immobilon-P transfer membrane (Millipore Company) in a transfer buffer, which contained 25 mM Tris-HCl, 120 mM glycine and 20% methanol (v/v) for the determination of relative protein contents with immunoblotting analysis. The transferred membranes were shaken for 2 hours in blocking buffer, including TBS (10 mM Tris-HCl, 150 mM NaCl solution) and 5% fat-free powdered milk, then incubated for 1 hr at room temperature with polyclonal anti-PKC-α, -β, -ε, -ζ, -θ, and -δ isozyme antibodies (1:1000 Life Technologies), respectively. The transferred membranes were subsequently incubated with biotinylated anti-rabbit IgG (1:5000; Amersham) for 40 min and then finally with streptavidin conjugated horseradish peroxidase (1:5000; Amersham) for 30 min. The blots were rinsed in the TBS-T (10 mM Tris-HCl, 150 mM NaCl and 0.2% Tween 20 solution) 3 times (5 min each time) between each of the preceding steps. For chemiluminescent detection, the membrane sheets were dipped into luminal substrate solution (Amersham) and the chemilumigrams were developed on Hyperfilm-ECL (Amersham) to visualize PKC isozymes. The normal exposure time ranged from 2 to 5 min. The relative content of PKC isozyme was determined by the model GS-670 Imaging Densitometer (Bio-Rad Company) with the Image Analysis Software Version 1.0. The relative protein

content for each experimental condition was expressed as a percentage of control value (control band density was considered as 100%).

7. Analysis of PKA protein content

The relative protein content of PKA was obtained by running 12% SDS-PAGE and Western blotting. The method used was similar to that for PKC isozyme analysis. The anti-PKA polyclone antibody was from Transduction Laboratories and the concentration of 1:1000 was utilized for the primary antibody. Cell lysates (5 μl) was used as a positive control in Western blotting experiments. It was supplied along with the anti-PKA antibody purchased. The cell lysate was derived from a pituitary tumor of a female Wistar-Furth rat and is an excellent positive control for PKA. It is provided at a concentration of 0.4 mg protein/ml in SDS-PAGE buffer.

8. Statistical analysis

Data were expressed as mean ± SE. Unpaired Student's *t* test was employed when comparing the difference between the control and the experimental group values. Probability P value < 0.05 was taken to represent a significant difference.

IV. RESULTS

1. Rat model of diabetic cardiomyopathy

A. General characteristics of diabetic and insulin-treated animals

In comparison to the control animals, the body weight and ventricular growth were significantly decreased whereas the ventricular to body weight ratio was increased in rats 8 weeks after the streptozotocin injection (Table 1). Daily injection of insulin to the diabetic animals for 2 weeks normalized the ventricular weight and ratio of ventricular to body weight; however, the insulin-treated diabetic rats still had lower body weight. The depressed plasma insulin level and elevated glucose level in diabetic animals were corrected upon treatment with insulin (Table 1). These characteristics are similar with those reported for diabetic animals from this laboratory and by others (23,176-178,185).

B. Cardiac PKC activity

PKC activity was measured in the homogenate, cytosolic and particulate fractions from control, diabetic and insulin-treated diabetic rat hearts. As shown in Figure 3, cardiac PKC activity in the diabetic group was increased by about 43% and 90% in the homogenate fraction and cytosolic fractions in comparison to their respective control values, respectively, whereas no significant change in cardiac

Table 1. General characteristics of control, diabetic and insulin-treated diabetic animals

	Control	Diabetic	Diabetic + Insulin
Body wt. (g)	554 ± 22.5	307 ± 16*	362 ± 25*
Ventricular wt. (mg)	1081± 95	852 ± 37*	922 ± 26†
Ventricular/body wt. (mg/g)	2.01± 0.06	2.78 ± 0.005*	2.55 ± 0.07†
Plasma glucose (mg/dl)	161± 9.5	496 ± 5.3*	193 ± 6.5†
Plasma insulin (μU/ml)	29.2 ± 3	12.5 ± 0.9*	35 ± 1.4†

Values are mean ± SE of 6 experiments. * Significantly different from control ($P < 0.05$);
† Significantly different from diabetic group ($P < 0.05$).

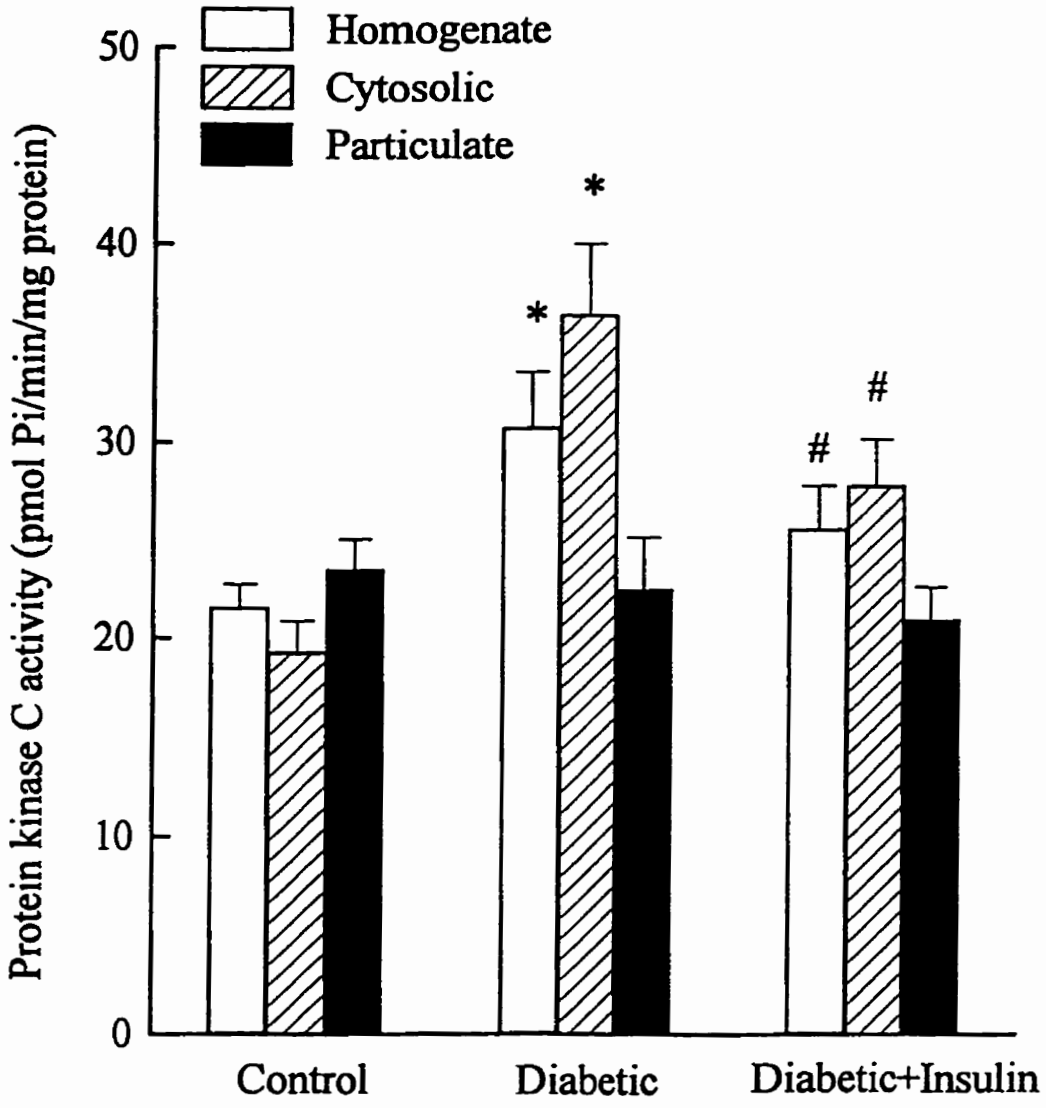

Figure 3. Cardiac PKC activity in diabetic cardiomyopathy in rat. Values are mean ± SE of 6 samples in each group. *P<0.05 compared with control, #P<0.05 compared with diabetic group.

PKC activity was found in the particulate fraction. The changes of PKC activities in homogenate and cytosolic fractions of diabetic rat hearts were partially reversed by insulin treatment (Figure 3).

C. Relative protein content of PKC isozymes

The protein content of PKC-α, -β, -ε, and -ζ isozymes in both cytosolic and particulate fractions of the cardiac muscle from control, diabetic and insulin-treated diabetic rats was identified by Western blotting. Typical bands representing the PKC-α, -β, -ε, and -ζ isozymes in both cytosolic and particulate fractions of rat hearts are shown in Figure 4. The densitometric analysis of PKC-α, -β, -ε, and -ζ isozymes revealed an increase of 125%, 500%, 260%, and 215% in relative protein contents in the cytosolic fraction of the diabetic heart in comparison to the control value, respectively (Figure 5 and 6). It may be noted that the increase in the relative protein content of PKC-β isozyme in cytosolic fraction from diabetic heart was largest (500%) whereas that of PKC-α isozyme was smallest (125%). Insulin administration to the diabetic rats resulted in partial normalization of the increased relative protein contents of all PKC isozymes in the cytosolic fraction (Figure 5 and 6). No significant alteration in PKC isozyme protein content of the particulate fraction from the diabetic heart was evident when compared to control value (Figure 5 and 6).

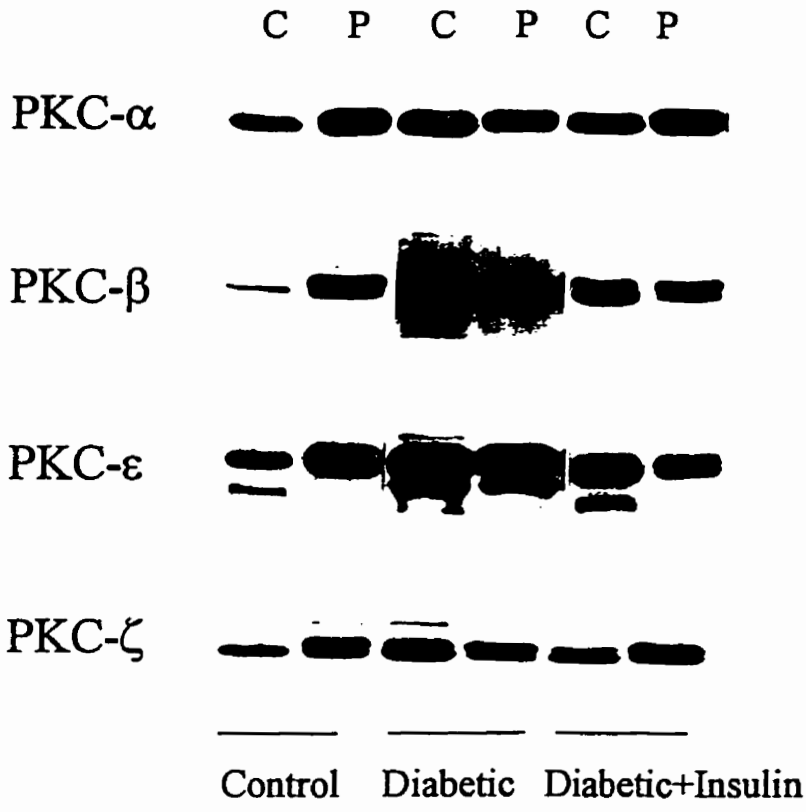

Figure 4. Typical immunoblot bands for cardiac PKC-α, -β, -ε, and -ζ isozymes in both cytosolic (C) and particulate (P) fractions from control, diabetic and insulin-treated diabetic groups.

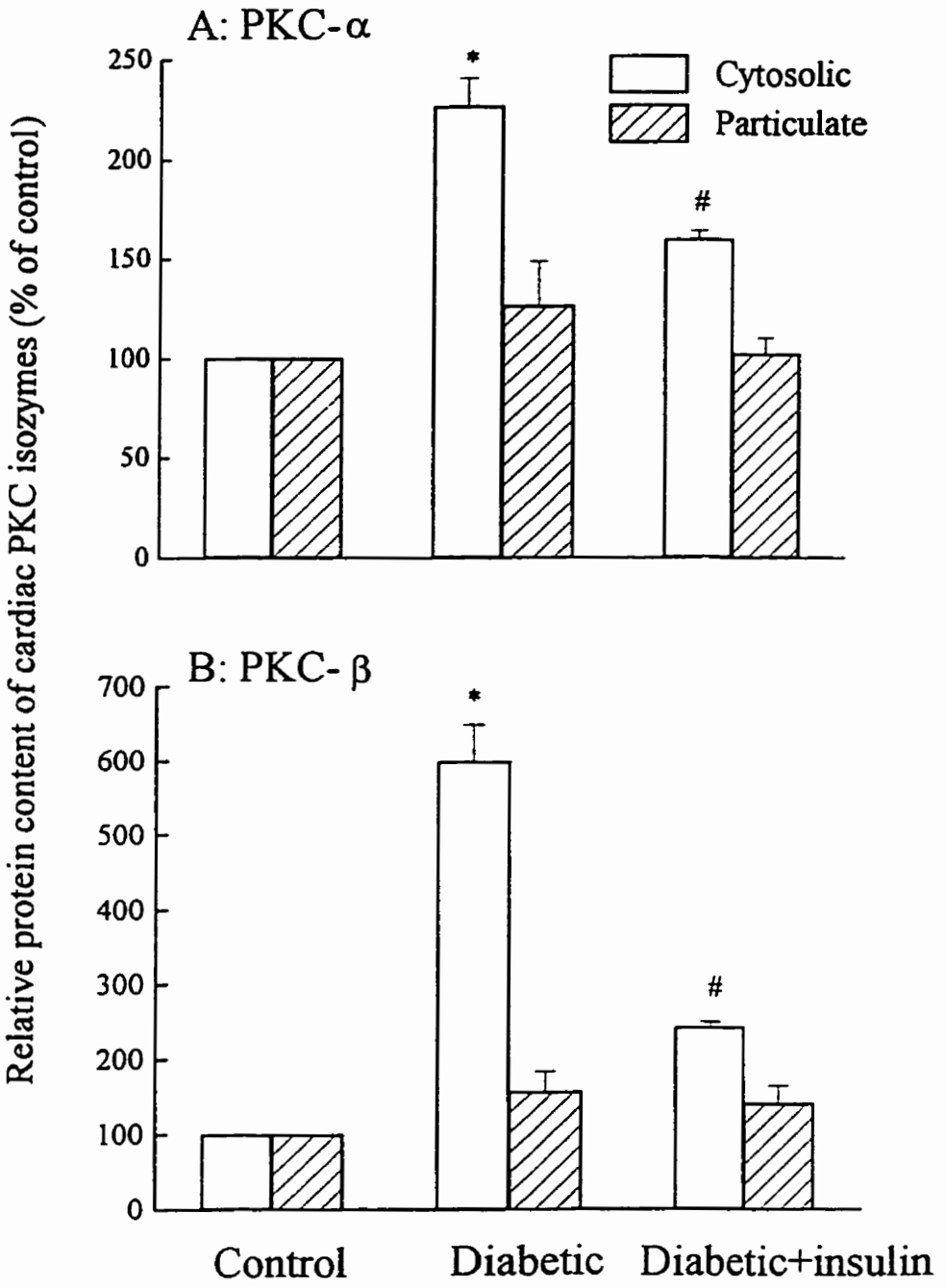

Figure 5. Analysis of relative protein content of cardiac PKC-α and PKC-β isozymes in cytosolic and particulate fractions from control, diabetic and insulin-treated diabetic groups. Values are mean ± SE of 6 samples in each group. *P<0.05 compared with control, #P<0.05 compared with diabetic group.

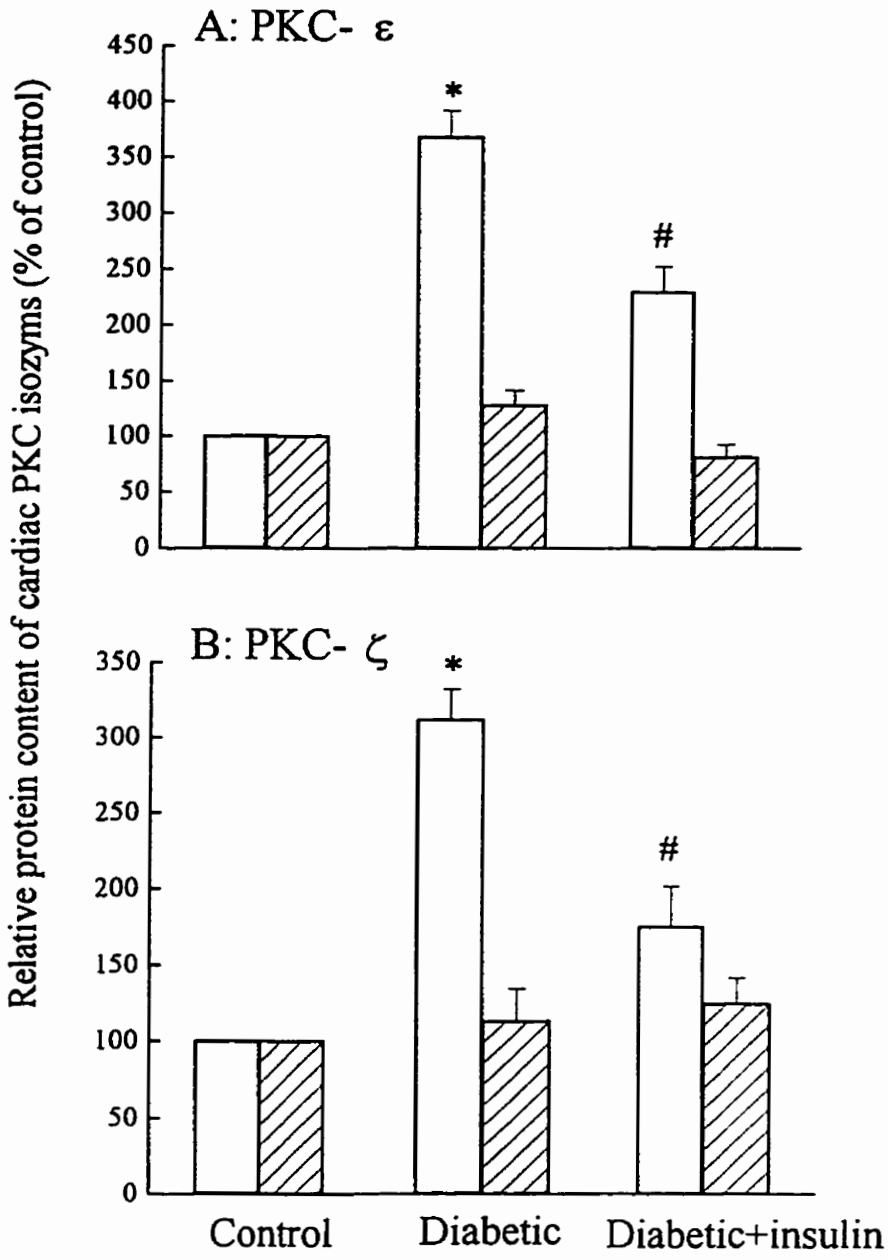

Figure 6. Analysis of relative protein content of cardiac PKC-ε and PKC-ζ isozymes in cytosolic and particulate fractions from control, diabetic and insulin-treated diabetic groups. Values are mean ± SE of 6 samples in each group. *P<0.05 compared with control, #P<0.05 compared with diabetic group.

2. Cardiomyopathic hamster model

A. General characteristics of cardiomyopathic animals

On the basis of clinical signs, the UM-X 7.1 strain of cardiomyopathic hamsters at the age of 250-300 days are classified at late stage of congestive heart failure (3,5). Results in Table 2 show increased ventricular wt, ventricular to body wt ratio and liver wt in cardiomyopathic hamsters in comparison to the control animals. The presence of ascites in cardiomyopathic hamsters employed in this study indicates the occurrence of congestive heart failure in these animals.

B. Cardiac PKC activity

Data in Figure 7 show that the specific PKC activity was significantly increased by about 260 to 280% in homogenate fraction, cytosolic fraction and particulate fraction in cardiomyopathic hamster hearts in comparison to control values. Significant increases in the cardiac PKC activity in all fractions were also evident when the results were expressed on the basis of enzyme activity per g heart wt.

Table 2. General characteristics of 250-300 days old control and cardiomyopathic hamsters

	Control	Cardiomyopathic
Body wt (g)	229 ± 14.5	261 ± 16.2
Ventricular wt (mg)	867 ± 5.1	1226 ± 4.9*
Ventricular/body wt ratio (mg/g)	3.8 ± 0.2	4.7 ± 0.1*
Liver wt (g)	6.1 ± 0.3	8.0 ± 0.2*
Ascites (ml)	ND	7.4 ± 0.4*

The observations are based on 16 animals for each group. Liver and heart wts represent the fresh tissue weights. * Significantly different from control value ($P<0.05$). ND: not detectable.

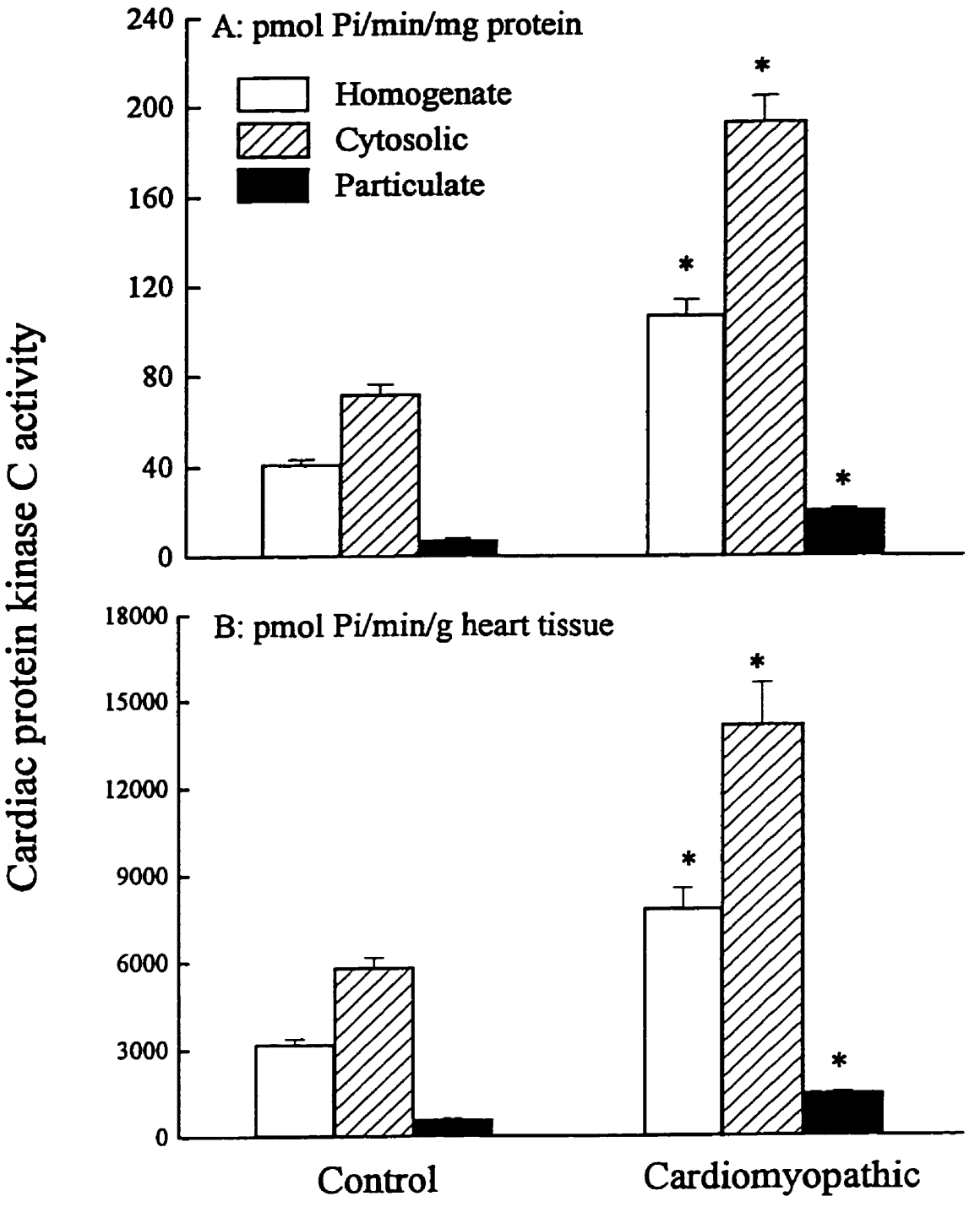

Figure 7. Cardiac PKC activity in control and cardiomyopathic hamsters expressed as A: pmol Pi/min/mg protein and B: pmol Pi/min/g heart tissue. Values are mean ± SE of 6 samples in each group. * $P<0.05$ compared with control.

C. Relative protein content of cardiac PKC isozymes

In order to the evaluate changes in different PKC isozymes, different cardiac PKC isozyme alterations were measured in the cardiomyopathic hamsters. Typical immunoblot bands for PKC-α, -β, -ε, and -ζ isozymes in control and cardiomyopathic hamster heart are shown in Figure 8. The relative protein content of the cardiac PKC-α in the cytosolic fraction was significantly increased (24%) when compared to the control value, without any significant change in the particulate fraction in the cardiomyopathic hamsters (Figure 9). On the other hand, the relative protein content for PKC-β was unaltered in both cytosolic and particulate fractions of the cardiomyopathic hearts (Figure 9). The results in Figure 10 indicate that the relative protein contents for PKC-ε, unlike those for PKC-ζ, were increased in both cytosolic and particulate fractions of the cardiomyopathic hearts. The use of antibodies for PKC-δ and PKC-θ did not reveal the presence of these forms of PKC isozyme in the hearts of control and cardiomyopathic hamsters.

D. PKC activity in skeletal muscle

To test if changes in PKC activity in cardiomyopathic hamsters were limited to the heart, PKC activity was also measured in the skeletal muscle. The results in Figure 11 indicate that the PKC activities in the homogenate, cytosolic and particulate fractions from the cardiomyopathetic skeletal muscles were markedly

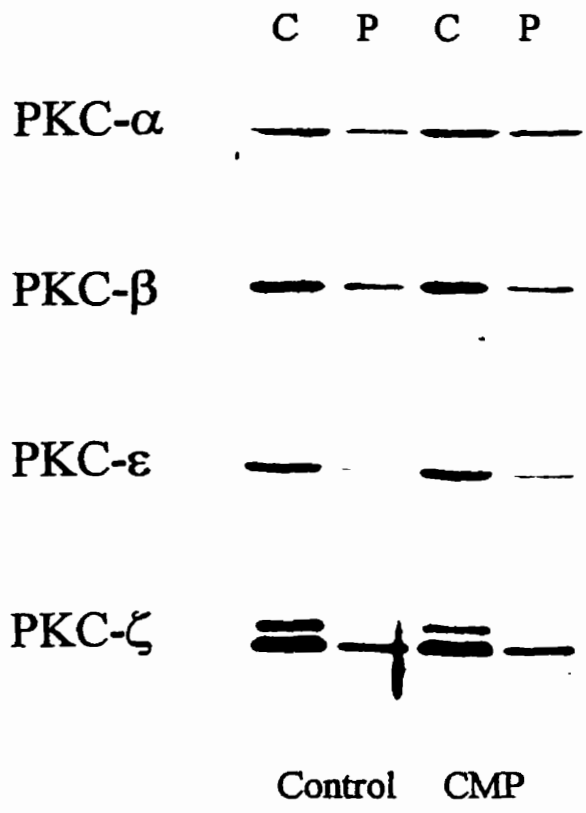

Figure 8. Typical immunoblot bands for cardiac PKC-α, -β, -ε, and -ζ isozymes in both cytosolic (C) and particulate (P) fractions from control and cardiomyopathic (CMP) hamsters.

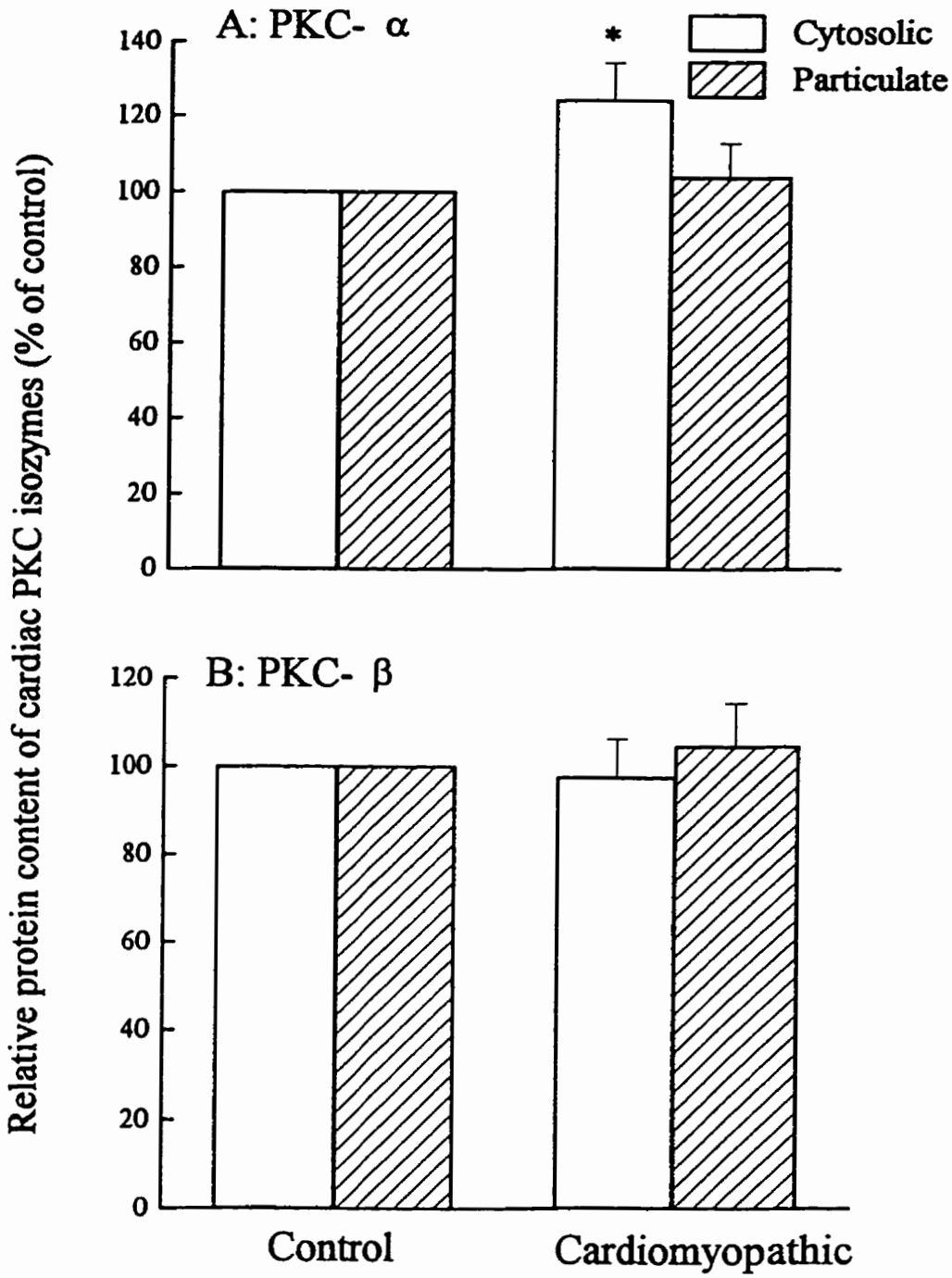

Figure 9. Analysis of relative protein content of cardiac PKC-α and PKC-β isozymes in cytosolic and particulate fractions from control and cardiomyopathic hamsters. Values are mean ± SE of 6 samples in each group. *P<0.05 compared with control.

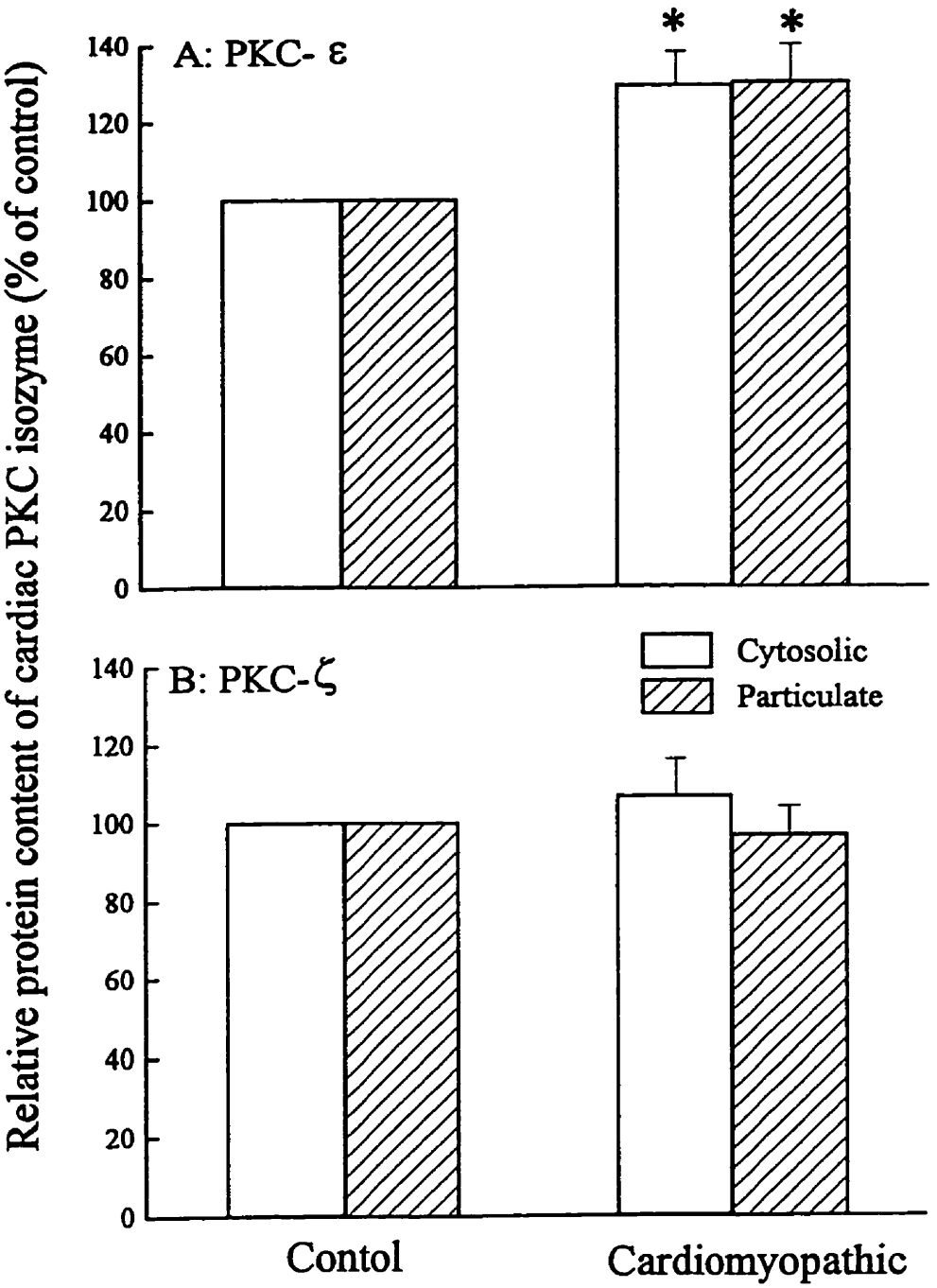

Figure 10. Analysis of relative protein content of cardiac PKC-ε and PKC-ζ isozymes in cytosolic and particulate fractions from control and cardiomyopathic hamsters. Values are mean ± SE of 6 samples in each group. *P<0.05 compared with control.

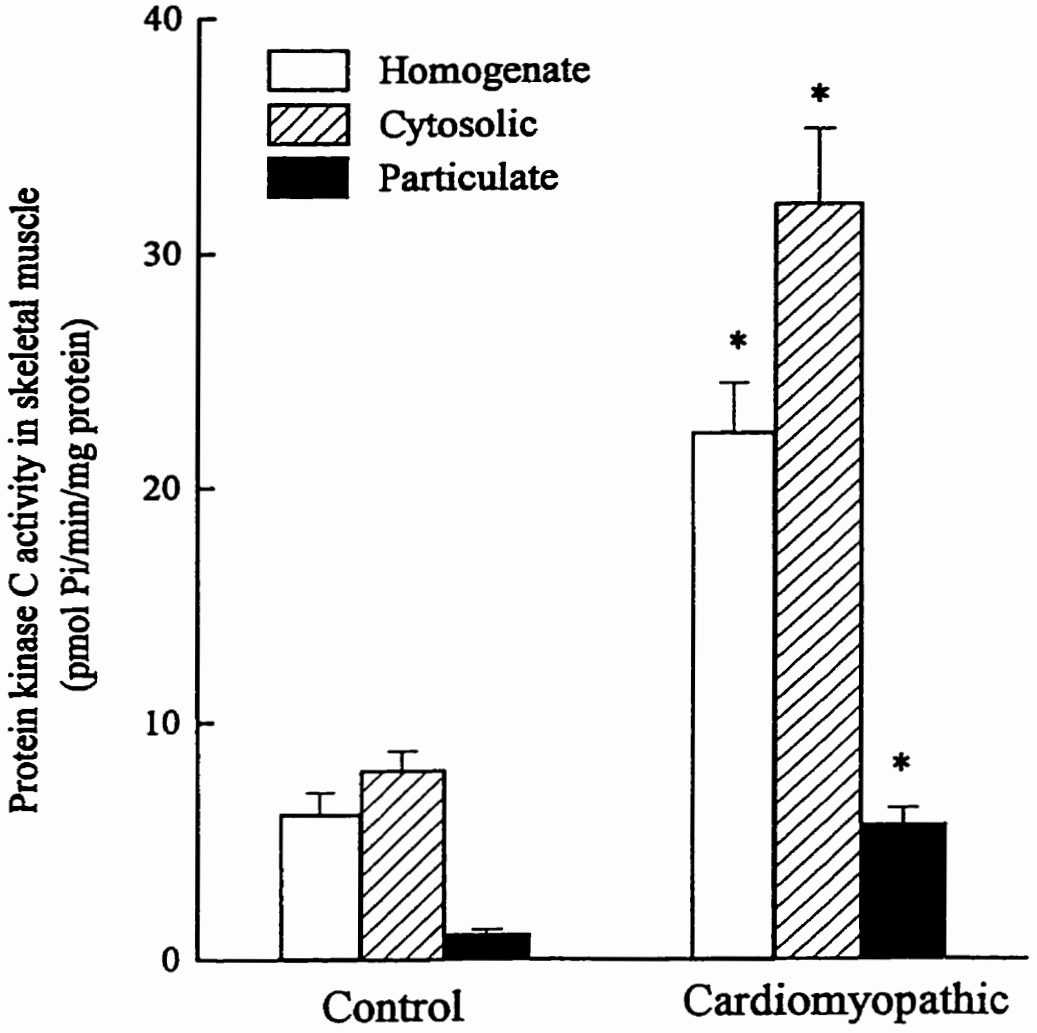

Figure 11. PKC activity in skeletal muscle from control and cardiomyopathic hamsters. Values are mean ± SE of 6 samples in each group. *P<0.05 compared with control.

increased. However, we were unable to detect the PKC isozymes of skeletal muscle by employing antibodies for different PKC isozymes.

E. Cardiac and skeletal muscle PKA activity and relative protein content

The specificity of PKA changes in cardiac and skeletal muscles from cardiomyopathic hamsters was studied by measuring PKA activity and protein content in these tissues. The results in Figure 12 indicate that the cardiac and skeletal muscle PKA activity in cardiomyopathic hamsters was significantly increased when compared to their respective control values (Figure 12). The relative protein content for PKA were measured by densitometric analysis of immunoblots obtained by antibodies for PKA. Since the PKA antibody used here was polyclonal, positive control was used to ensure that the bands analyzed were truly of protein kinase A. The data in Figure 13 reveal that the relative protein content for PKA were increased by 45% and 50% in cardiac and skeletal muscles from the cardiomyopathic hamsters, respectively.

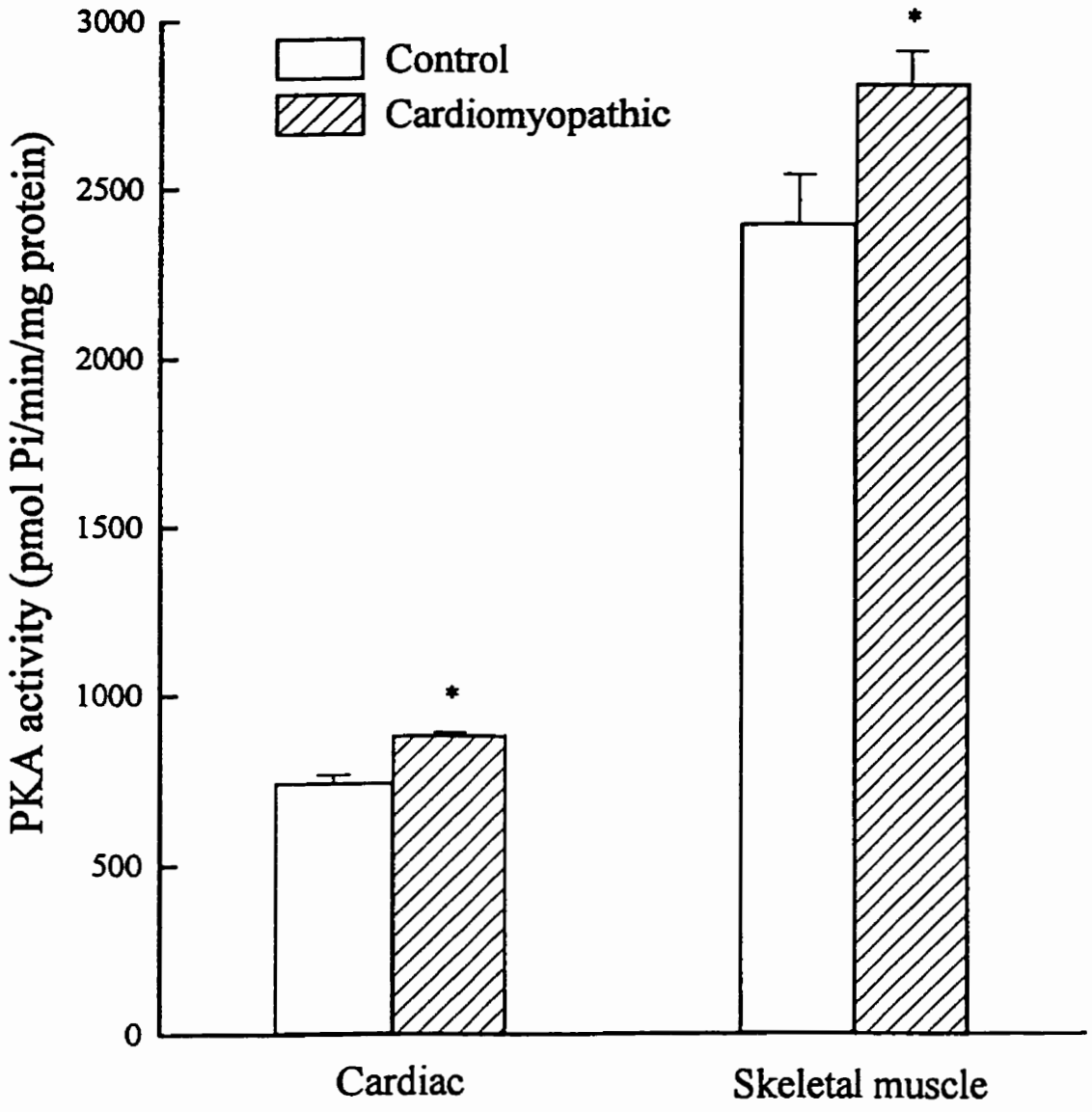

Figure 12. Cardiac and skeletal muscle PKA activities in control and cardiomyopathic hamsters. Values are mean ± SE of 6 samples in each group. *P<0.05 compared with control.

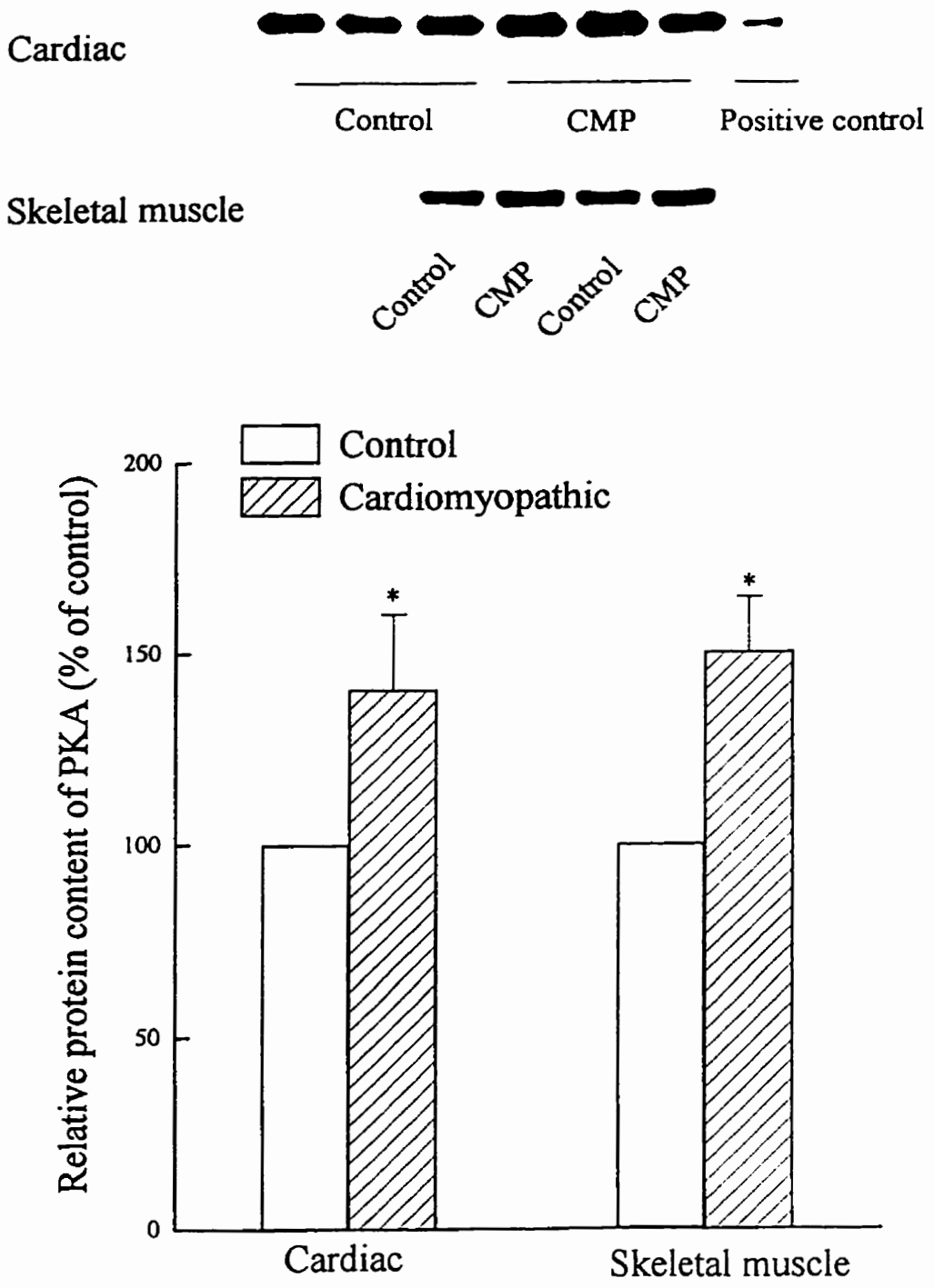

Figure 13. Immunoblots and densitometric analysis of relative protein content of cardiac and skeletal muscle PKA in control and cardiomyopathic (CMP) hamsters. Values are mean ± SE of 6 samples in each group. *P<0.05 compared with control.

V. DISCUSSION

1. Diabetic cardiomyopathy

Hyperglycemia is an important etiologic factor in the development of macrovascular and microvascular diseases in diabetes. One of the possibilities in this regard lies in the activation of PKC since it may participate in the regulation of vascular permeability, contractility, hormone receptor turnover, and proliferation (14,186). Although the exact mechanism of PKC activation in diabetic smooth muscle is poorly understood, several reports have indicated that the total DAG levels are increased in vascular tissues and cells in culture in response to diabetes (14,187). In the present study, we here demonstrated a significant increase in PKC in the homogenate fraction of hearts from diabetic rats induced by an intravenous injection of streptozotocin. Such an increase in cardiac PKC was found to be primarily due to PKC present in the cytosolic rather than particulate fraction of the diabetic heart. The exact mechanism underlying the increase in the cytosolic PKC in diabetic heart still remains unexplored. While an increase in the de novo synthesis of DAG has been reported as a result of hyperglycemia in the diabetic myocardium (187,188), it is interesting that no additional recruitment of PKC to the membrane occurs. Since only membrane-bound PKC is enzymatically active, these observations suggest diabetes is not associated with enhanced PKC-mediated signalling. It may be,

however, that one of the consequences of the increase in DAG level in the diabetic heart as a result of hyperglycemia, like that proposed for diabetic smooth muscle (189), may be an increase in PKC levels present in diabetic heart as observed in this study. An influence on gene expression as a mechanism for the increase in cardiac PKC, whether mediated by DAG or not, is nevertheless one consequence of diabetes.

Abnormal myocardial function in the diabetic rat heart is often related to the defective cardiac contraction due to depressed ATPase activities of actomyosin and myosin as well as a shift in myosin heavy chain to β-subtype (94,178,185). Since PKC is known to phosphorylate troponin I and troponin T subunits with dissociation of Ca^{2+} from troponin C and depressed cardiac contraction (91), it is possible that the observed increase in PKC may play an important role in the depression of cardiac function in diabetes. Furthermore, PKC-β isozyme has been demonstrated to be responsible for myosin heavy chain shift (from α to β), which has the lower myosin ATPase activity than that of α subtype. Since PKC-β isozyme was found to exhibit the largest increase (500% to control) in protein content, it is suggested that PKC-β isozyme may be a major factor for the increase in PKC and heart dysfunction in diabetic cardiomyopathy.

Insulin deficiency in diabetes is associated with a marked activation of the sympathetic nervous system and an elevation of circulating catecholamines (10). An increase in sympathetic activity can be seen to promote an increase in intracellular

concentration of Ca^{2+} in the diabetic heart. Diabetic cardiomyopathy has also been shown to be associated with intracellular Ca^{2+} overload resulting from membrane defects (6). Since PKC activity is modulated by Ca^{2+} (51), it is possible that the increase in PKC cytosolic, especially the PKC-α and PKC-β isoforms, in diabetes result from the increase in the intracellular concentration of Ca^{2+}. On the other hand, PKC may contribute to the Ca^{2+} overload in diabetic cardiomyopathy via phosphorylation of L-type Ca^{2+} channels in the sarcolemmal membrane (47,190). Nonetheless, the alterations in cardiac PKC observed in this study are due to insulin deficiency in streptozotocin-treated rats (14). This view is based on our observation that in vivo treatment of diabetic rats with insulin partially normalized the cytosolic PKC content in the heart. The partial normalization of PKC enzymes by insulin treatment for 2 weeks may be due to a short duration of the treatment per se.

2. Genetic cardiomyopathy in hamsters

Our results demonstrated that only PKC-α, -β, -ε, and -ζ were present, while PKC-δ, and -θ were not detectable in control and cardiomyopathic UM-X 7.1 hamster hearts. Previous reports have indicated a considerable variability in the PKC isozyme expression in mammalian hearts. Using immunoautoradiography, Cai et al. (147) found that PKC-α, -ε, and -ζ isozymes were present in Syrian BIO 14.6 hamster heart. Gu and Bishop (115) showed that the PKC-α, -β, -ε, -δ, and -ζ

isozymes existed in control and hypertrophic rat hearts induced by pressure overload. On the other hand, Rybin and Steinberg (191) demonstrated that PKC-ε and PKC-δ were present in cardiaomyocytes, whereas PKC-α and PKC-ζ were present in nonmyocytes and PKC-β was totally absent in the rat heart. These discrepancies in results in the cardiac content of PKC isozymes in various studies may be due to the methods of isolation and detection as well as assay conditions, specificity of isozyme antibodies and potential species differences.

In the present study we have demonstrated that the cardiac PKC levels were significantly increased in both cytosolic and particulate fractions at late stages of congestive heart failure in the genetic cardiomyopathic hamsters. This increase in cardiac PKC was correlated with an elevation of cytosolic PKC-α, and PKC-ε isozymes as well as of particulate PKC-ε. Since the hypertrophic cardiomyopathic hamster hearts are known to exhibit elevated activities of phosphatidylinositide metabolism with markedly enhanced release of arachidonic acid and DAG (15,192), the observed changes in cardiac PKC may be due to increased level of DAG as well as changes in gene expression for PKC proteins in cardiomyopathic heart. It should also be noted that arachidonic acid is known to be a potent activator of both PKC-ε and PKC-ζ and thus may be a potential cause of enhanced PKC isozyme protein content (56,58). Although the exact contribution of increased PKC to heart function in the cardiomyopathic hamster may not be readily apparent, it is suggested that the

increased PKC may influence both cardiac adaptation and cardiac decompensation. Since the secretion of ANP is increased by the stimulation of PKC activity (119), the elevated levels of cardiac PKC in cardiomyopathic hamster may enhance ANP release and thus produce beneficial effects on heart function indirectly through the action of ANP on blood vessels and kidney. Furthermore, in view of the involvement of PKC in increasing RNA content of cultured cardiomyocytes and stimulating proto-oncogene expression for the development of cardiac hypertrophy (101), expression of the immediate early genes during the course of cardiomyopathy may be due to PKC activation. On the other hand, several myocardial regulatory proteins including C-protein, troponin I, and troponin T are stoichiometrically phosphorylated by PKC in vitro (91). Since phosphorylation of troponin I or T by PKC results in a decreased Ca^{2+}-dependent MgATPase activity in reconstituted actomyosin (91), it is possible that depressed myofibrillar ATPase activity and heart dysfunction in cardiomyopathic hamsters may be due to elevated levels of cardiac PKC activity. Thus the increase in PKC may play both the compensatory and decompensatory roles in changing heart function during the development of cardiomyopathy in hamsters (193).

The results reported in this study show that the increase in PKC was not specific because another signal transduction protein, PKA, was also elevated in cardiomyopathic hearts. There is a report (151) showing no change in the PKA activity of human failing myocardium; however, the difference between this and our

results may be due to the methods used for the activity assay as well as due to the fact that all the patients with heart failure received various kinds of treatment, including cardiac glycosides, diuretics and nitrates, which may influence the primarily pathological condition. A prior study in our laboratory regarding the failing heart in UM-X 7.1 cardiomyopathic hamsters showed an uncoupling of β-adrenoceptors (5), which may be due to the observed increase in PKA, which has been shown to uncouple the β-adrenoceptors in the myocardium. It should be mentioned that PKC and PKA usually relay information along different signal pathways within the cell, but these two enzymes sometimes lead to similar cellular events. These protein kinases often react with the same phosphate substrates and even phosphorylate the same seryl and threonyl residues in a single protein molecule (53). Recently, PKC-activating phorbol esters have been shown to selectively stimulate some isozymes of adenylyl cyclase in the HT4 neural cell line (194). Phorbol esters synergistically stimulate cAMP formation when adrenaline receptors were simultaneously activated, indicating a conditional activation of cAMP production by phorbol esters. Our data showing increases in PKA and PKC in the cardiomyopathic hamster heart support the concept of cross-talk between PKA and PKC. The specific interaction between PKC and PKA has also been observed in platelets, hepatocytes, adipocytes, and lymphocytes (194-198).

Although cardiomyopathy is not usually a major feature of patients with skeletal muscle abnormalities, the possibility of cardiac involvement in these

patients has been recognized. On the other hand, some cardiomyopathic patients have been reported to exhibit a skeletal muscle myopathy (36,37,199). The abnormalities of both cardiac and skeletal muscle are common in terms of histologic findings including endomysial fibrosis and increased lipid deposits (38,200). Such findings indicate the coexistence of a generalized myopathy in patients with cardiomyopathy. While the cause of the generalized myopathy is not known, both PKC and PKA were found to be increased in cardiac and skeletal muscle in cardiomyopathic hamsters. It should be noted that skeletal muscle metabolism in familial cardiac hypertrophy has been documented as being dependent upon the contractile protein affected (200). In inherited hypertrophic cardiomyopathy, the phenotype has been linked to the β-myosin heavy chain (MHC) gene. It has been shown that normal and mutant cardiac β-MHC gene message is also expressed in skeletal muscle of cardiomyopathic patients with identified mutations in the β-MHC gene (201). Since PKC-β is known to play a role in β-MHC shift, it is quite possible that PKC-β may participate in the cardiomyopathic pathophysiology. Skeletal muscle is enriched for the mRNA encoding PKC-α and PKC-θ, suggesting that these are the most abundant PKC isoforms present in the skeletal tissue (202,203). But in our study, PKC isoforms were not detectable using the same antibodies employed for the analysis of cardiac tissue.

VI. SUMMARY AND CONCLUSIONS

1. The cardiac PKC levels in homogenate and cytosolic fractions were significantly elevated in diabetic cardiomyopathy and these changes were partially normalized by insulin treatment.
2. The relative content of cytosolic PKC-α, -β, -ε, and -ζ was increased in the diabetic heart, and these changes were partially reversed by insulin treatment.
3. No significant changes in total cardiac PKC levels or the levels of specific PKC isoforms was observed in the particulate fraction of diabetic rat heart.
4. The cardiac PKC levels in both cytosolic and particulate fractions were significantly increased in the failing heart due to genetic cardiomyopathic hamsters.
5. The relative amount of cytosolic PKC-α and PKC-ε and that of the particulate PKC-ε was increased in the ventricles from cardiomyopathic hamsters.
6. The PKC levels in both cytosolic and particulate fractions were significantly augmented in skeletal muscle from cardiomyopathic hamsters.
7. Both cardiac and skeletal muscle PKA content was elevated in the failing hearts of cardiomyopathic hamsters.
8. The increase in cytosolic cardiac PKC corresponded with the elevation of PKC-α, -β, -ε, and -ζ isoforms in diabetic cardiomyopathy, and the therapeutic effect of insulin may be partly through PKC pathway.

9. The increase in PKA and PKC due to congestive heart failure may relate to the increase in PKA and PKC-α and PKC-ε isozymes, and these alterations may contribute to the skeletal muscle and cardiac dysfunctions in genetic cardiomyopathic hamsters.

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IMAGE EVALUATION
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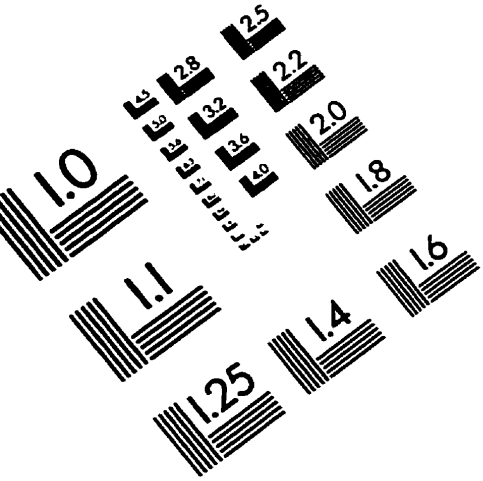

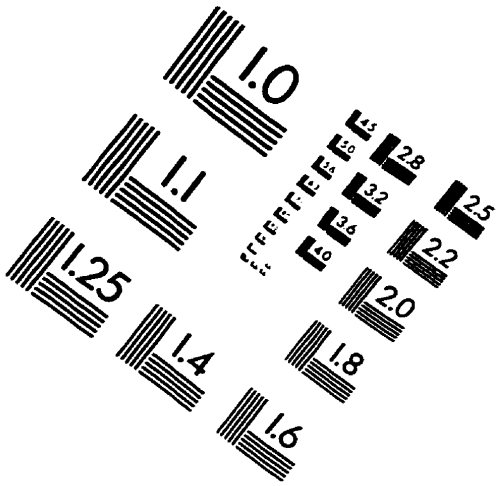

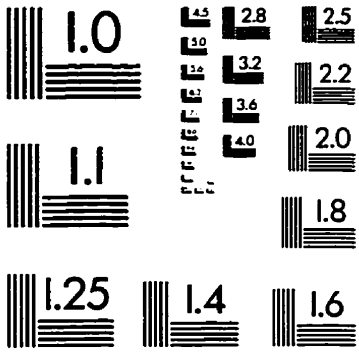

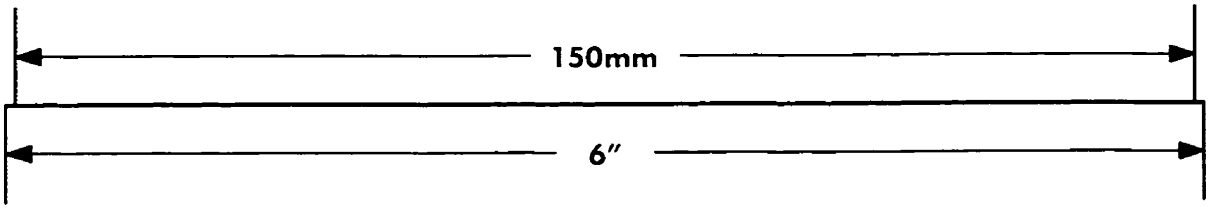

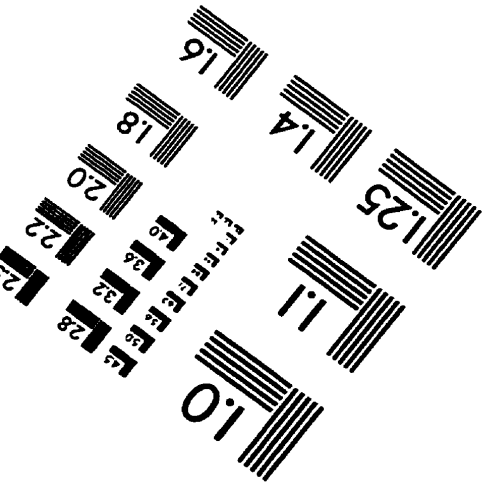

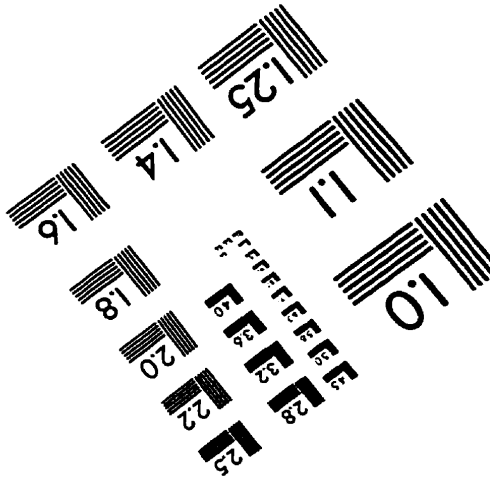

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