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Research Article

Regulation of muscle creatine kinase by phosphorylation in normal and diabetic hearts

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Abstract. Protein kinase C (PKC) is an important signaling molecule in the heart, but its targets remain unclear. Using a PKC substrate antibody, we detected a 40-kDa phosphorylated cardiac protein that was subsequently identified by tandem mass spectroscopy as muscle creatine kinase (M-CK) with phosphorylation at serine 128. The forward reaction using ATP to generate phosphocreatine was reduced, while the reverse reaction using phosphocreatine to generate ATP was increased following dephosphorylation of immunoprecipitated M-CK with protein phosphatase 2A (PP2A) or PP2C. Despite higher PKC levels in diabetic hearts, decreased phosphorylation of M-CK was more prominent than the reduction in its expression. Changes in CK activity in diabetic hearts were similar to those found following dephosphorylation of M-CK from control hearts. The decrease in phosphorylation may act as a compensatory mechanism to maintain CK activity at an appropriate level for cytosolic ATP regeneration in the diabetic heart.

Keywords. Muscle creatine kinase, PKC isoform, phosphorylation, ATP, phosphocreatine.

Introduction

Creatine kinase (CK), which is found in at least three isoforms in all vertebrates, catalyzes the reversible conversion of creatine plus ATP to phosphocreatine and ADP [1]. The brain (B-CK) and muscle (M-CK) isoforms exist as homo- and heterodimers in the cytosol, and their function is to prevent fluctuations of ATP during periods of high energy demand, such as in cardiac and skeletal muscle contraction, Ca^{2+}-pump activity, photoreceptor-mediated light transduction, and neuronal excitation. Mitochondrial forms (Mt-CK) are usually found as octamers, which function to transfer high-energy phosphates from ATP to creatine in mitochondria to form phosphocreatine, which is shuttled to the cytosol where high-energy phosphates are transferred back to ATP by M-CK for cellular energy consumption [2].

The structure, catalytic mechanism and kinetics of CK have undergone intensive investigation for more than 50 years. However, only a few studies have investigated the regulation of this enzyme. These have suggested that CK is subjected to both transcriptional and translational regulation as well as post-translational modification under different metabolic conditions. For example, expression of the M-CK isoform has been found to be reduced in diabetic cardiomyopathy and heart failure. At the post-translational level, several kinases have been found to modify the activity of CK, presumably through phosphorylation. For instance, PKC was shown to phosphorylate B-CK in chicken brain [3] and in mouse skin cells, resulting in a small increase in its activity [4, 5], while AMP-

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activated protein kinase (AMPK) was shown to phosphorylate M-CK, resulting in a reduction of its enzymatic activity in skeletal muscle [6]. Recently, it was suggested that multiple kinases and phosphatases may be involved in the phosphorylation and dephosphorylation of CK in skeletal muscle from hibernating ground squirrels [7]. In the failing heart, post-translational modification was also suggested to regulate CK activity, but the nature of the regulation is unclear [8]. Kinase-mediated phosphorylation site(s) were not identified in any of these previous studies.

Diabetic cardiomyopathy is a common complication observed in both human patients and experimental animal models with type 1 and type 2 diabetes, and is associated with abnormalities of contractility, energy metabolism and calcium homeostasis [9, 10]. Increased activation of protein kinase C (PKC) has been shown to be closely associated with diabetic cardiovascular complications [11, 12]. While PKCβ_2 is the isoform most commonly reported to increase in cardiovascular tissues from diabetic animals, activation of PKCε has also been reported in the heart [13–17]. However, despite intensive investigation in recent years, potential downstream targets of PKC phosphorylation in the heart are not well defined. In this study, we applied a combination of biochemical analysis, mass spectroscopy and measurements of enzyme activity to identify and characterize potential PKC phosphorylation targets in hearts from normal rats and rats treated with streptozotocin (STZ), which produces a form of poorly controlled type 1 diabetes associated with the development of cardiomyopathy. We found that M-CK is a major target of PKC phosphorylation in hearts from normal rats. Furthermore, we identified the amino acid sequence of the PKC phosphorylation site in M-CK and characterized its potential role in modulation of M-CK activity. Unexpectedly, we found a reduced level of phosphorylation of this enzyme in diabetic hearts despite similar or increased levels of PKC isoforms compared to control hearts. This was associated with an increase in M-CK activity in the reverse direction generating ATP using phosphocreatine.

Material and methods

Induction of diabetes. Male Wistar rats (200–250 g) were anesthetized with isoflurane and given a single tail vein injection of 60 mg/kg STZ in 0.1 M citrate buffer (pH 4.5) or citrate buffer alone as control. STZ-treated rats with blood glucose levels over 13 mmol/l 1 week after injection were considered diabetic. After 12–14 weeks, tissues were obtained from animals for experiments. Control and diabetic rats were deeply anesthesized with sodium pentobarbital (65 mg/kg, i. p.) and hearts and other tissues were immediately removed and frozen in liquid nitrogen. The use of animals in this investigation conformed to the Canadian Council on Animal Care Guidelines on the Care and Use of Experimental Animals, and all protocols were approved by the University of British Columbia Animal Care Committee.

Tissue homogenates. Frozen tissues were powdered and homogenized in buffer containing 20 mM Tris-HCl (pH 7.5), 50 mM 2-mercaptoethanol, 5 mM EGTA, 2 mM EDTA, 10 mM NaF, 1 mM PMSF, 25 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1 % NP40, 0.1 % SDS, 0.1 % deoxycholic acid and 1 % phosphatase inhibitor cocktail (Sigma). The tissue homogenates were spun at 700 *g* for 5 min to remove debris and unbroken cells, and the protein content of the supernatants was determined by the Bradford protein assay. The samples were frozen in small aliquots at –70°C for further experiments.

Western blot analysis. Equal amounts of protein (60 μg) from control and diabetic hearts, or protein phosphatase 2A (PP2A)-treated and untreated protein (30 μg) were separated by 10 % SDS PAGE and transferred to PVDF membrane. The membranes were blocked for 1 h in a solution of 5 % skim milk and then incubated overnight at 4°C with a 1:1000 dilution of anti-PKC α, β_2, δ or ε (Santa Cruz, CA), a 1:1000 dilution of anti-PKCα phospho T638 (Epitomics, CA), anti-PKCβ_2 phospho T641, anti-PKCδ phospho T505 (Cell Signaling Technology, MA), anti-PKCε phospho S729 (Abcam, MA) or anti-phospho-(Ser) PKC substrate (Cell Signaling Technology), a 1:2000 dilution of anti-M-CK or anti-B-CK, a 1:500 dilution of anti-Mit CK (Santa Cruz Biotechnology, CA), or a 1:10 000 dilution of GAPDH (Santa Cruz). Membranes were washed and incubated with secondary antibody conjugated to horseradish peroxidase (1:10 000) for 1 h. Protein bands were identified by a standard ECL method (Amersham Inc, Québec, Canada). For quantification of M-CK, the concentration in heart samples was calculated from the intensity of the M-CK band in reference to a standard curve constructed from known concentrations of purified M-CK.

Tandem mass spectroscopy (MS/MS). The candidate proteins were identified by Western blotting with anti-phospho-(Ser) PKC substrate antibody. The bands were excised from the gel and were subjected to in-gel digestion with the trypsin profile IGD kit (Sigma) according to the manufacturer's protocol. Phosphopeptides were isolated from total peptides using a MonoTip pipette tip according to the manufacturer's instructions (GL Sciences, CA). The resulting peptides were analyzed with an API Q STAR PULSARi

Hybrid LC/MS/MS at the Proteomics Core Facility of the University of British Columbia, and a subsequent database search was performed with Mascot software.

Enzymatic activity assays. The forward reaction (production of phosphocreatine) was determined by oxidation of NADH to NAD^+ at 340 nm, coupled to pyruvate kinase and lactate dehydrogenase [18]. The assay buffer contained 5 mM ATP, 50 mM creatine, 130 mM KCl, 6 mM $MgCl_2$, 0.4 mM PEP, 15 U/ml LDH, 7 U/ml PK and 15 mM Tris (final pH 8.8). For the reverse reaction (production of ATP), the activity was determined by NADPH formation at 340 nm, coupled to hexokinase and glucose-6-phosphate dehydrogenase [19, 20]. The assay buffer included 1.5 mM ADP, 2 mM AMP, 50 μM DiAPP, 5 mM glucose, 130 mM KCl, 1 mM $MgCl_2$, 0.7 mM $NADP^+$, 9 mM phosphocreatine, 0.5 U/ml glucose-6-phosphate dehydrogenase, 1.3 U/ml hexokinase, and 10 mM Tris (final pH 7.4). Muscle or heart tissue extract was diluted 1:10 to 1:50 with 130 mM KCl, 26 mM Tris (pH 7.4), 1 % NP40, and 20 mM 2-mercaptoethanol and 10 μl of the diluted sample was used in each assay. Readings were taken every minute. The calculations were based on molar extinction coefficient of 6270 at 340 nm for NADH and NADPH. The rate was taken from the linear portion of the curve.

Dephosphorylation by PP2A or PP2C. Anti-M-CK antibody was incubated with 500 μg protein from control hearts, and then was immunoprecipitated with the Catch and Release® kit 2 (Upstate, Lake Placid, NY) according to the manufacturer's instructions. The protein samples obtained were incubated without and with 0.5 U PP2A or PP2C (Upstate) for 60 min at room temperature. The resulting samples were then used for assay of CK activity in both the forward and reverse directions, as well as Western blotting with the anti-phospho-(Ser) PKC substrate and anti-M-CK antibodies as described above.

Phosphorylation of M-CK by PKC isoforms and kinetic study. Purified M-CK (Sigma, 2 μg) was incubated with equal activity of each PKC isoform (Upstate) in a total volume of 25 μl of the reaction mixture, including 0.4 mM ATP and other competitive metabolites as indicated, at 37°C for 45 min. The reaction mixture contained 20 mM Tris pH 7.4, 0.03 % Triton-X 100, 0.5 mM $CaCl_2$, 2.5 mM glycerol 2-phosphate, 1 mM DTT and 2 mM $MgCl_2$. At the end of the incubation, SDS gel loading buffer (5 μl) was added and the samples were boiled for 5 min. Proteins were separated by SDS-PAGE and transferred to PVDF membrane. Phosphorylated M-CK was detected by Western blot with anti-phospho-(Ser) PKC substrate antibody (Cell Signaling Technology). For kinetic analysis of the phosphorylation of M-CK by PKCδ, various concentrations of M-CK substrate (1, 2, 4, 8 and 16 μM) were combined with 91.8 μU PKCδ (1 μl) in the reaction mixture above and were incubated at 37°C for 45 min. At the end of the incubation, samples were processed for Western blot with anti-phospho-(Ser) PKC substrate antibody as described above. Optical densities of phosphorylated M-CK were determined and expressed as OD units of the product per unit PKCδ per hour. The data were then fitted to either the Michaelis–Menten equation or the Hill equation (below) using GraphPad Prism software, to obtain kinetic parameters.

$$v = \frac{V_{max}[S]^h}{[S]^h + K_{0.5}^h}$$

where $K_{0.5}$ is the apparent K_m and h is the Hill coefficient.

Phosphatase activity determination. Phosphatase activity was determined in homogenates of control and diabetic hearts using a commercially available kit (RediPlate 96 EnzChek® serine/threonine phosphatase assay kit; Molecular Probes) according to the manufacturer's instructions.

Statistics. All values are expressed as means ± SEM. Statistical analysis was performed using the Student's unpaired *t*-test for comparison of two groups, or one-way ANOVA followed by the Newman-Keuls test when more than two groups were compared, using GraphPad Prism (GraphPad Software). For all results the level of significance was set at $p<0.05$.

Results

All diabetic rats had blood glucose levels greater than 22 mmol/l, and exhibited other symptoms of diabetes, including polydipsia, polyuria, increased food intake, and reduced body weight. We have previously found that these animals develop typical characteristics of diabetic cardiomyopathy, including reduced left ventricular developed pressure and rates of pressure development and decline (+dP/dt and –dP/dt) of isolated working hearts *ex vivo*, and significantly reduced percent fractional shortening, an index of systolic function, *in vivo* [21].

PKC phosphorylation in heart. Western blotting of total cellular protein from control and diabetic hearts with an antibody selectively recognizing phospho-

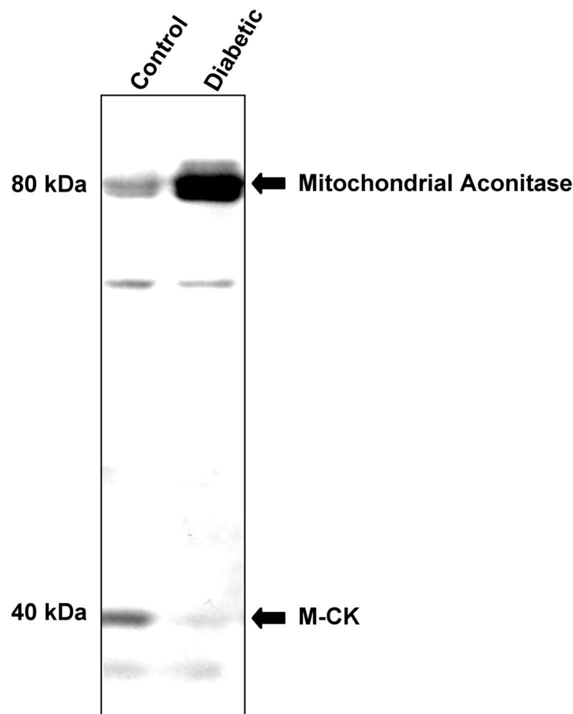
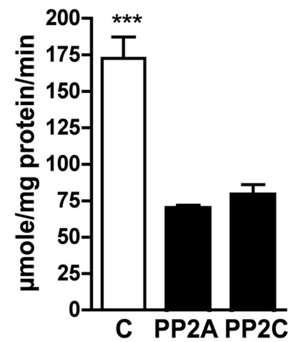
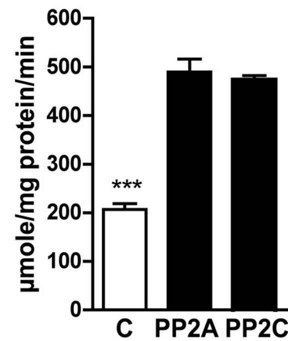


Figure 1. Protein kinase C (PKC) phosphorylated proteins in hearts from control and diabetic rats. Representative Western blot showing detection of 80- and 40-kDa phosphorylated proteins with anti-phospho-(Ser) PKC substrate antibody in hearts from control and diabetic rats.





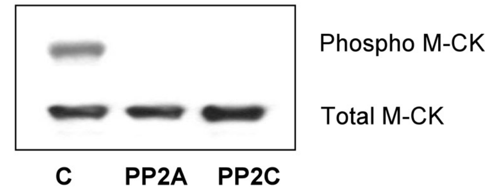


Figure 2. The effect of dephosphorylation on creatine kinase (CK) activity. The forward (top) and reverse (middle) activities of CK were measured after dephosphorylation of immunoprecipitated protein (C) from control hearts with protein phosphatase 2A (PP2A) or PP2C for 60 min at room temperature. The activities were expressed according to total protein used in the immunoprecipitation; n=5 in each group; *** p<0.001 compared to all. Representative Western blot with anti-phospho-PKC substrate and anti-M-CK antibodies, confirming dephosphorylation of M-CK and equal amount of protein loaded in each lane (bottom).

serine PKC substrate motifs revealed two bands, one at 40 kDa and the other at 80 kDa, with altered phosphorylation (Fig. 1). The corresponding 40-kDa band obtained from control heart samples was excised and subjected to in-gel trypsin digestion for protein identification by MS/MS. M-CK was identified in the 40-kDa band by Mascot search against the Swiss-Prot database with the resulting peptide sequences. Phosphopeptides were then isolated from in-gel trypsin-digested peptides from the same band and subjected to MS/MS analysis. A single phosphopeptide [GGDDLDPNYVLS(p)SR] along with 20 other non-phosphopeptides was detected and matched by Mascot search to M-CK. No peptides found were matched to either B-CK or Mt-CK. The phosphorylation site was identified as Ser128 in M-CK. We also identified the 80-kDa protein as mitochondrial aconitase (m-aconitase, aconitase 2) and detailed characterization of this enzyme is being carried out in a separate study.

Effect of dephosphorylation on CK activity. To investigate the functional consequences of the phosphorylation of M-CK, the enzyme was immunoprecipitated from control hearts using anti-M-CK antibody, and the activity of the enzyme determined either without further treatment, or following treatment with PP2A or PP2C. The results showed that the activity of the forward reaction (creatine + ATP → phosphocreatine + ADP) was reduced significantly (Fig. 2, top) while the reverse reaction (ADP + phosphocreatine → ATP + creatine) was doubled after dephosphorylation with either PP2A or PP2C (Fig. 2 middle). This indicates that the phosphorylation of CK indeed regulates the activity of the enzyme. In parallel, confirmation of the dephosphorylation of the immunoprecipitated M-CK by the phosphatases is shown in Figure 2.

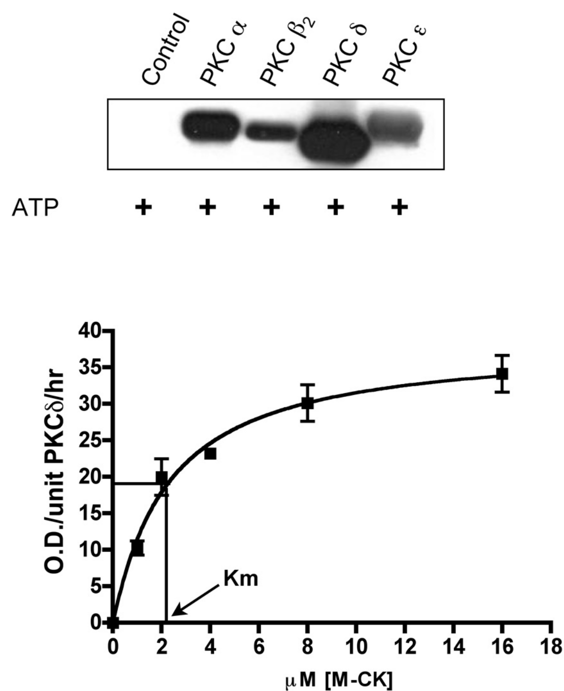


Figure 3. Phosphorylation of muscle creatine kinase (M-CK) by PKC *in vitro.* The same amount of purified M-CK was incubated with equal activity of each purified PKC isoform in presence of 0.4 mM ATP at 37°C for 45 min. Protein was separated by SDS-PAGE and phosphorylated M-CK was detected by Western blot with anti-phospho-(Ser) PKC substrate antibody (top). ODs of phosphorylated M-CK *versus* the concentration of M-CK substrate phosphorylated of M-CK by PKCδ were fitted to the Michaelis--Menten equation with GraphPad Prism to determine kinetic parameters (below). Data were averaged from four individual experiments.

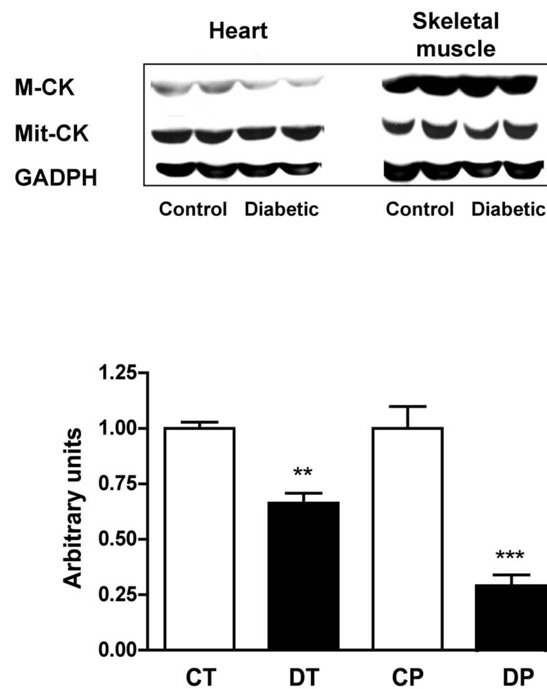


Figure 4. CK isoform expression in hearts and skeletal muscles from control and diabetic rats. M-CK and mitochondrial CK (Mt-CK) expressions (above) in total protein from heart (left panel) and skeletal muscles (right panel) by Western blotting (top). The first two lanes represent the samples from control rats and last two lanes represent the samples from diabetic rats. GAPDH loading control shown below. Quantification of total M-CK normalized to GAPDH, and phosphorylated M-CK from Figure 1 (bottom). CT and DT represent total M-CK, while CP and DP represent phosphorylated CK from control and diabetic hearts, respectively; $n=5$ in each group, ** $p<0.01$ compared to control total group; ***$p<0.001$ compared to control phosphorylated group by one-way ANOVA.

***In vitro* phosphorylation of M-CK by PKC.** To identify potential isoform-specific phosphorylation, purified M-CK was incubated with each of the three major cardiac PKC isoforms, PKCα, δ and ε, as well as with $PKC\beta_2$ *in vitro* and phosphorylated M-CK was then detected by Western blotting with the PKC substrate antibody. The results showed that equi-active levels of all four PKC isoforms were able to phosphorylate M-CK *in vitro* (Fig. 3 top). PKCδ appeared to the most active isoform in phosphorylation of M-CK. To investigate the kinetics of the phosphorylation of M-CK by PKCδ, various concentrations of M-CK substrate were phosphorylated with a fixed amount of PKCδ and levels of phosphorylated M-CK were determined by Western blotting. Micheaelis-Menten analysis of the resulting data showed that the K_m and V_{max} were 2.2 μM and 38 OD units of phosphorylated M-CK per unit PKCδ per hour, respectively (Fig. 3, bottom). Analysis of the data by non-linear curve-fitting using the Hill equation showed that the Hill coefficient was equal to one, suggesting that there were no cooperative effects in the phosphorylation of M-CK by PKCδ .

CK expression, phosphorylation and activity in diabetic hearts. Phosphorylation of the 40-kDa protein in hearts of 12–14-week diabetic rats was much less than that from control animals (Fig. 1). To investigate the basis for the change in phosphorylation status of M-CK from diabetic hearts, we first determined total protein expression of M-CK by Western blotting. The expression of this enzyme was reduced by 34% in diabetic hearts compared to control (Fig. 4, top left panel). By comparing to a standard curve of known concentrations of purified M-CK, the levels of M-CK in control and diabetic hearts were found to be 29.8±4.2 μM and 19.7±5.1 μM, respectively (mean ± SD, $n=10$; $p=0.0001$, Student's unpaired *t*-test). In contrast, no difference in the expression of Mt-CK was found, while we only detected a trace amount of B-CK in hearts from both control and diabetic rats. The magnitude of

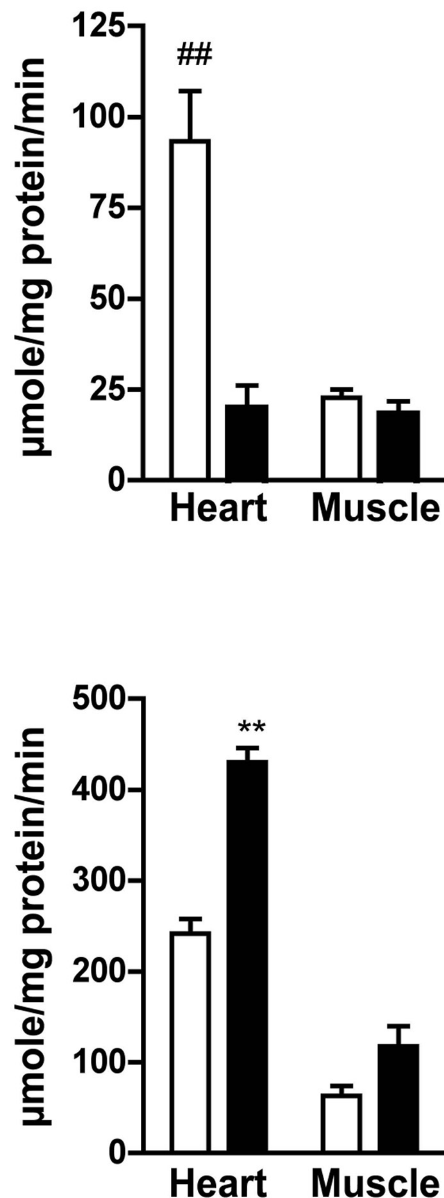


Figure 5. CK activity in heart and skeletal muscle from control and diabetic rats. The forward (top) and reverse (below) activities of CK from control (open bars) and diabetic rats (black bars) animals. ## $p<0.01$ compared to diabetic group. ** $p<0.01$ compared to control by unpaired *t*-test.

decrease in phosphorylation of M-CK, which was reduced by 71.2±0.05 % in diabetic hearts compared to control (Fig. 4, below), was much greater than the decrease in expression of the enzyme. To determine whether similar changes in protein expression and phosphorylation were found in other tissues, we also determined PKC phosphorylated targets by Western blotting with the same antibody recognizing phosphoserine PKC substrate motifs, as well as the expression of M-CK and Mt-CK isoforms in skeletal muscle from control and diabetic rats. However, no differences in PKC phosphorylated targets (data not shown) or expression of CK isoforms were found in skeletal muscle from diabetic rats compared to control (Fig. 4, top right panel).

CK activity in both the forward and reverse directions was also compared in protein extracted from control and diabetic hearts. The activity of the CK forward reaction was 80 % lower than control (Fig. 5, top), while the activity of the reverse reaction was increased by about 70 % based on total cellular protein (Fig. 5, bottom). These changes were entirely consistent with those found following dephosphorylation of CK from control hearts (Fig. 2). If the lower levels of M-CK protein were used to normalize the activity, the reverse activity was about 2.5-fold higher in diabetic than in control hearts. In contrast, CK activity was similar in both directions in skeletal muscle from control and diabetic rats (Fig. 5). The increase in the reverse activity of CK in hearts from diabetic rats increases the transfer of high-energy phosphate from phosphocreatine to ATP.

PKC isoform expression and its phosphorylation. We next determined whether decreased phosphorylation of M-CK in diabetic hearts could be attributed to lower expression of any of the major isoforms of PKC. Levels of total and T638-phosphophorylated PKCα as well as total and T505-phosphorylated PKCδ were unchanged (results not shown), while expressions of total and T641-phosphorylated PKCβ_2 as well as total and S729-phosphorylated PKCε were significantly increased in diabetic hearts compared to control (Fig. 6). Therefore, the decreased phosphorylation of M-CK in diabetic hearts was not associated with a decrease in the expression or phosphorylation of the major cardiac PKC isoforms.

Phosphatase activities in control and diabetic hearts. The possibility that the decreased phosphorylation of M-CK in diabetic hearts was due to increased phosphatase activity was also investigated. However, the activities of the two major cardiac phosphatases, PP1 and PP2A, were found to be similar between control and diabetic hearts (Fig. 7).

Energy metabolites and phosphorylation of M-CK by PKC *in vitro*. We next investigated whether energy metabolites could modulate the phosphorylation of M-CK by PKC *in vitro*. The phosphorylation of M-CK by equi-active amounts of purified PKCβ_2 or PKCδ added with ATP alone or with additional 5'-AMP, 5'-ADP, phosphocreatine or creatine was determined. The results showed that the greatest reduction of M-CK phosphorylation was produced by 5'-AMP (Fig. 8,

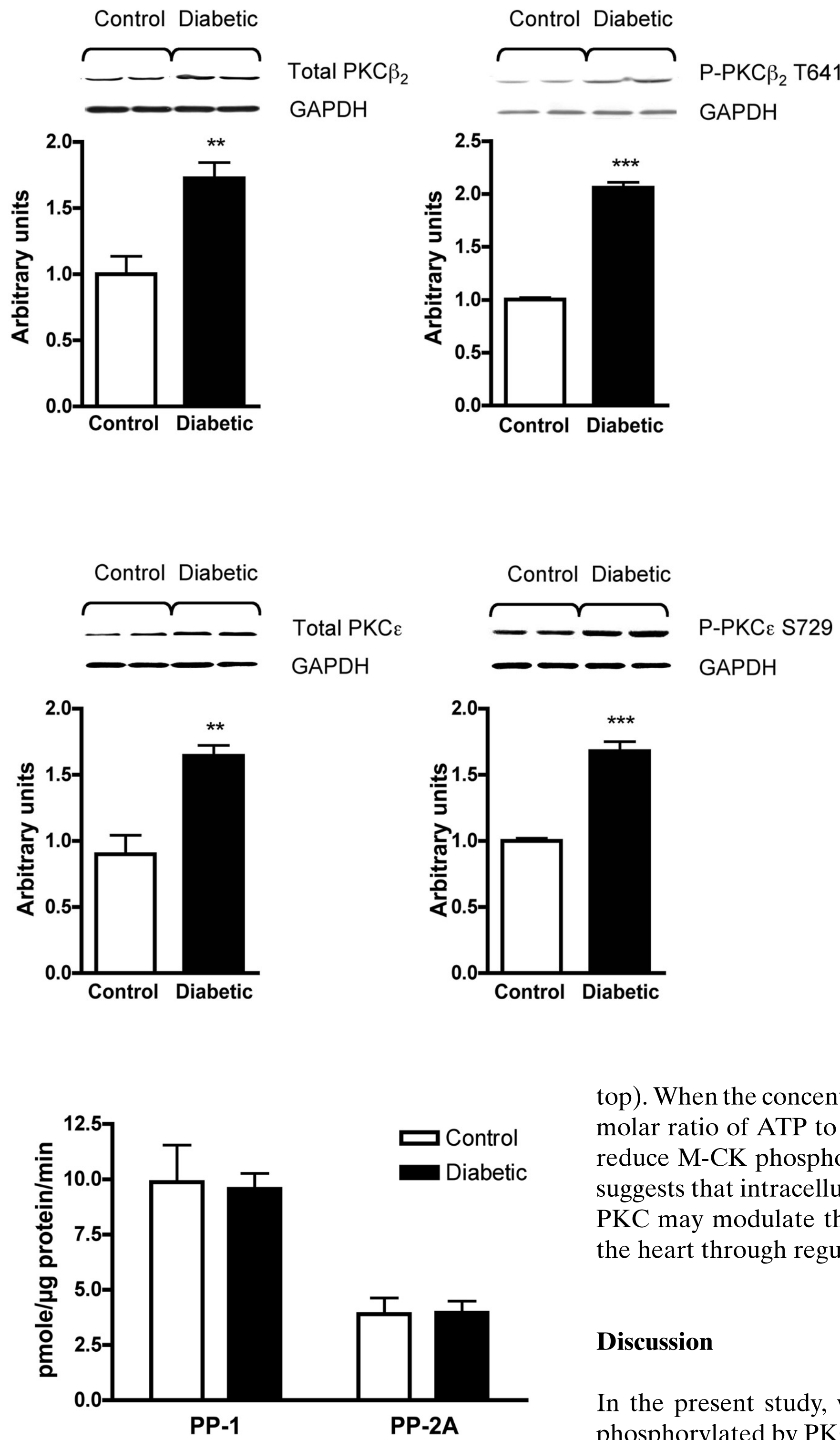


Figure 6. Total and phospho-PKC isoforms from control and diabetic hearts. Representative Western blots with total cellular protein are shown here. Mean ODs of each isoform of total PKC and the corresponding phosphorylated form normalized to their corresponding GAPDH loading control are shown; $n=5$ in each group, ** $p<0.01$, *** $p<0.001$ by unpaired *t*-test.

Figure 7. Activities of protein phosphatases PP1 and PP2A in hearts from control and diabetic rats. $n=8$ in each group. There were no significant differences between control and diabetic hearts in the activity of either enzyme.

top). When the concentration of 5'-AMP was varied, a molar ratio of ATP to AMP of 10 or less was able to reduce M-CK phosphorylation (Fig. 8, bottom). This suggests that intracellular ATP/AMP ratio along with PKC may modulate the reverse activity of M-CK in the heart through regulation of its phosphorylation.

Discussion

In the present study, we demonstrate that M-CK is phosphorylated by PKC both *in vivo* and *in vitro*. The phosphorylation site was identified as Ser128, which is predicted to be the PKC phosphorylation site by the algorithm developed by Blom et al. [22]. Phosphorylation of Ser128 in M-CK reduces the reverse activity in normal hearts, while dephosphorylation of the serine residue increases the transfer of high-energy

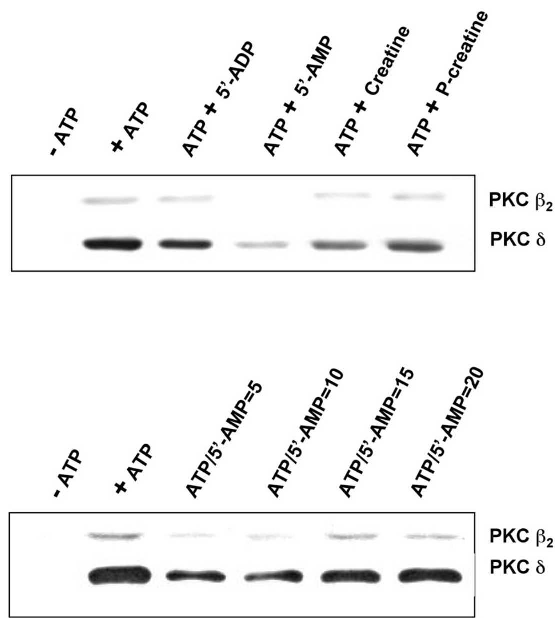


Figure 8. Effect of metabolites and the ATP/AMP ratio on phosphorylation of M-CK *in vitro*. Effect of different metabolites (0.4 mM each) on M-CK phosphorylation by PKCβ_2 or PKCδ in presence of 0.4 mM ATP (top). Effect of a fixed concentration of AMP (0.4 mM) with an increasing ratio of ATP to AMP on M-CK phosphorylation by PKCβ_2 and PKCδ (below). Representative results are shown; similar results were obtained when experiments were repeated at least four times.

phosphate from phosphocreatine to ATP in the reverse reaction in hearts from diabetic rats.

Previous studies have suggested that CK activity is regulated by PKC in chicken brain [3] and in skin cells [4, 5], and by AMPK [6] and multiple kinases [7] in skeletal muscle. In addition, recently a reduction in M-CK phosphorylation was found in muscles from aged compared to young rats [23]. However, the phosphorylation sites were not identified in these studies. It has also been reported that autophosphorylation of several threonine residues, including Thr224, Thr274, Thr282, Thr289, Thr322 and Thr327 occurs in M-CK following prolonged incubation of the enzyme [24]. None of these phosphorylation sites was detected in M-CK in either normal or diabetic hearts by MS/MS in the current study. It is not known whether autophosphorylation of these amino acids occurs *in vivo*, or if it is of physiological significance.

The major PKC isoforms expressed in normal rat heart are PKCα, PKCδ and PKCε, while PKCβ_2 has also been detected in a number of studies. It is not clear which of these isoforms is responsible for the phosphorylation of M-CK *in vivo* in normal hearts, since all four of these PKC isoforms were found to phosphorylate M-CK *in vitro*. However, dephosphorylation of M-CK from control heart with PP2A or PP2C *in vitro* produced a marked increase in the CK reverse activity while reducing the forward reaction, suggesting that PKC-mediated phosphorylation of M-CK regulates its activity *in vivo*.

The catalytic mechanism of CK has undergone intensive investigation by a combination of X-ray crystallography and site-directed mutagenesis, and amino acids involved in substrate binding and catalysis have been identified in recent years [1]. The nucleotide phosphate-binding pocket of CK consists of five highly conserved arginines (residues 130, 132, 236 292 and 320) that provide positive charges to interact with the negative charges of phosphate groups from the nucleotide [1]. Using the 3-D model for M-CK (Swiss-Model Repository, SMR P00564 and the Swiss-PdbViewer), we find that Ser128, which is located adjacent to Arg130, is also exposed at the

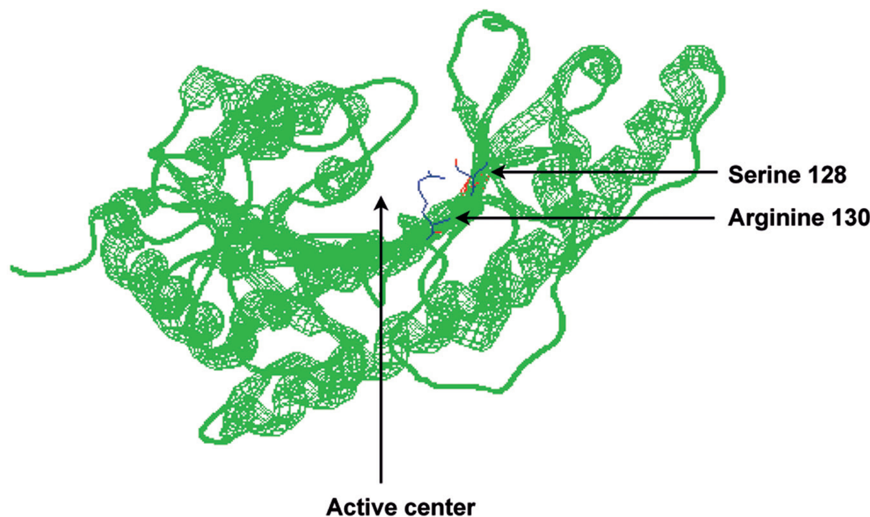


Figure 9. Interaction of phosphoserine 128 and arginine 130 on 3-D model of M-CK. Coordinates used were obtained from Swiss-Model Repository, SMR P00564.

surface of the molecule (Fig. 9). Thus, Ser128 would be easily accessible to PKC upon its activation. Once phosphorylated, the negative charge on the serine side chain may affect the positive charges on the neighboring Arg130, altering nucleotide binding and/or orientation within the active center, leading to altered catalytic activity of the enzyme.

Intracellular M-CK protein levels vary dramatically in different tissues, with a level of 31 μmol/kg being reported in normal rat heart [25]. This is similar to the result that we obtained in hearts from control rats in the present investigation, while M-CK was reduced by 34 % to 19.7 μM in diabetic hearts. Surprisingly, there is little information about the effects of diabetes on M-CK protein levels in the literature. Although one study reported that the M-CK mRNA expression was reduced by more than 60 % following 2 months of diabetes, the enzyme level was not determined [26]. Despite the reduction in M-CK protein levels, we found a 70 % increase of the reverse reaction of the enzyme in the current study. A 50 % increase in CK activity in left ventricles from 8-week diabetic rats was also reported [27]. However, a number of other studies reported a moderate reduction in CK activity, of the order of 25–30 %, in hearts from 4- or 8-week diabetic rats. This was presumably measured in the reverse direction (phosphocreatine to ATP), although the assay methods for CK activity were not specifically stated [26, 28, 29]. The velocity of the reverse CK reaction measured by ^{31}P-NMR was also found to be reduced by about 30 % in intact hearts in two of the studies [28, 29]. The reasons for the inconsistent findings among different groups are not entirely clear. Variable degrees of dephosphorylation of M-CK from control hearts processed in the absence of phosphatase inhibitors could have obscured a difference between control and diabetic hearts in CK activity measured *in vitro* in the earlier studies. However, this would not explain the lower reaction velocities obtained in intact diabetic hearts by ^{31}P-NMR. Another possibility is that it is only with increasing duration and severity of disease that dephosphorylation of M-CK takes place, leading to changes in its activity.

We have investigated several possibilities that may contribute to decreased M-CK phosphorylation in diabetic hearts. These included changes in PKC levels or phosphatase activity, as well as possible effects of decreased M-CK levels on the PKC kinetics. Consistent with previous reports demonstrating increased activation of PKCβ_2 and PKCε but not PKCα and PKCδ in hearts from diabetic rats [11, 17], we found increased expression and phosphorylation of PKCβ_2 and PKCε in diabetic hearts, with no change in expression or phosphorylation of PKCα or PKCδ, suggesting that changes in PKC activity were unlikely to be responsible for decreased phosphorylation of M-CK. Since activities of both PP1 and PP2A were reported to be higher in hearts from 1- to 4-week diabetic Sprague-Dawley rats [30], we then determined activities of the major cardiac phosphatases PP2A and PP1. However, there were no differences in the activity of these phosphatases in hearts from our diabetic model. Furthermore, kinetic analysis of the phosphorylation of M-CK by PKCδ showed that, despite the slight reduction in M-CK levels in diabetic hearts, its concentration was still well above the PKCδ K_m, while the Hill coefficient was equal to one, suggesting that there was no cooperativity occurring. The last possibility examined was whether an energy metabolite could interfere with phosphorylation of MCK by PKC. The results showed that the 5'-AMP to ATP ratio greatly affected the *in vitro* phosphorylation of M-CK by PKC. A molar ratio of ATP to 5'-AMP less than 10 was sufficient to reduce M-CK phosphorylation *in vitro* while other adenine nucleotides as well as creatine and phosphocreatine had little effect. It is possible that as the duration of diabetes is prolonged, mitochondrial ATP production in the heart becomes so impaired that 5'-AMP levels begin to increase. If the 5'-AMP levels were to increase in diabetic hearts, PKC-mediated phosphorylation of M-CK would then decrease, and the resulting increase in the reverse CK reaction would increase the conversion of phosphocreatine to maintain ATP levels. This mechanism remains to be further investigated as, to our knowledge, there is no information available about the effect of prolonged STZ-induced diabetes on 5'-AMP levels in the heart, although an ATP to 5'-AMP ratio of 4.5 was reported in hearts from BB-Wistar diabetic rats [31].

Elevation of 5'-AMP levels in the heart causes activation of AMPK, which was demonstrated to phosphorylate M-CK *in vitro* and in a muscle cell line, resulting in inhibition of the reverse CK reaction and reduced ATP formation [6]. However, further examination of this mechanism in the intact rat heart revealed that under normoxic conditions, an increase in workload was associated with increased 5'-AMP levels and AMPK reaction velocity, but with an increase rather than a decrease in the velocity of the CK reverse reaction [32]. These data suggest that AMPK itself does not inhibit CK activity in the reverse direction in the heart under these conditions. Although the mechanism responsible for the increased velocity of the reverse CK reaction with increasing workload was not determined, the results are consistent with our finding that an increase in 5'-AMP relative to ATP under high workload decreased PKC-mediated phosphorylation of M-CK, and that the latter would lead to increase the rate of the reverse reaction.

In summary, the present study demonstrates that M-CK is phosphorylated on Ser128 in normal hearts. The phosphorylation of the amino acid residue regulates the activity of the enzyme, reducing the transfer of a high-energy phosphate group from phosphocreatine to ATP in normal hearts. Dephosphorylation of M-CK in hearts from diabetic rats promotes the reverse activity of CK, increasing velocity of high-energy phosphate transfer from phosphocreatine to ATP. This may act as a compensatory mechanism to satisfy ATP requirements in the diabetic heart.

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