Role of p90 Ribosomal S6 Kinase (p90RSK) in Reactive Oxygen Species and Protein Kinase C β (PKC-β)-mediated Cardiac Troponin I Phosphorylation*

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Protein kinase C (PKC)-induced phosphorylation of cardiac troponin I (cTnI) depresses the acto-mysin interaction and may be important during the progression of heart failure. Although both PKCβII and PKCε can phosphorylate cTnI, only PKCβ expression and activity are elevated in failing human myocardium during end-stage heart failure. Furthermore, although increased cTnI phosphorylation was observed in mice with cardiac-specific PKCβ II overexpression, no differences were observed in cTnI phosphorylation status between wild type and cardiac-specific PKCε overexpression mice. A potentially important downstream effector of PKCs is p90 ribosomal S6 kinase (p90RSK), which plays an important role in cell growth by activating several transcription factors as well as Na+/H+ exchanger. Since both Ser24 and Ser44 of cTnI are contained in putative consensus sequences of p90RSK phosphorylation sites, we hypothesized that p90RSK is downstream from PKCβ II and can be a cTnI (Ser24/24) kinase. p90RSK, but not ERK1/2 activation, was increased in PKCβII overexpression mice but not in PKCε overexpression mice. p90RSK could phosphorylate cTnI in vitro with high substrate affinity but not cardiac troponin T (cTnT). To confirm the role of p90RSK in cTnI phosphorylation in vivo, we generated adenovirus containing a dominant negative form of p90RSK (Ad-DN-p90RSK). We found that the inhibition of p90RSK prevented H2O2-mediated cTnI (Ser24/24) phosphorylation but not ERK1/2 and PKCα/βII activation. Next, we generated cardiac-specific p90RSK transgenic mice and observed that cTnI (Ser24/24) phosphorylation was significantly increased. LY333,531, a specific PKCβ inhibitor, inhibited both p90RSK and cTnI (Ser24/24) phosphorylation by H2O2. Taken together, our data support a new redox-sensitive mechanism regulating cTnI phosphorylation in cardiomyocytes.

There is increasing support for the idea that excessive production of reactive oxygen species (ROS) contributes to the pathogenesis of diabetes. In particular, a strong correlation has been made between increased ROS, activation of specific protein kinase C (PKC) isoforms, and many functional consequences of diabetes (1, 2). This correlation has been strengthened by previous transgenic experiments in the heart, where overexpression of the PKCβ isoform, but not PKCε, decreased cardiac function (3–5). These data also suggest that distinct PKC isoforms may play differential functional roles in cell signaling pathways leading to cardiac dysfunction, but the exact significance of individual isoforms is not yet known.

The family of PKCs includes at least 11 isoforms (α, β, βII, γ, δ, ε, ζ, η, θ, λ, μ), representing the major downstream targets for lipid second messenger or phorbol esters (6, 7). PKC isoforms are classified into classical PKCs (α, β, βII, γ), which are Ca2⁺-dependent and contain two cysteine-rich zinc-finger-like motifs (C1 region) that bind diacylglycerol or phorbol ester and a Ca2⁺/phospholipids binding domain (C2 region). Novel PKCs (δ, ε, ζ, η, θ, μ) are diacylglycerol-sensitive but Ca2⁺/phospholipid-independent because of the absence of the C2 region, and atypical PKCs (ζ, λ) are rather insensitive to diacylglycerol as they lack one cysteine-rich motif in their C1 region but can be activated by phorbol ester. Our colleagues have previously reported that in failing human myocardium with end-stage heart failure, the expression and activity of Ca2⁺/sensitive PKCα and -β isoforms are elevated (8). In addition, cardiac-specific overexpression of PKCβII isoform in mice causes left ventricular hypertrophy, myocardial fibrosis, and decreased in vivo left ventricular performance (4). Note, these PKCβII transgenic mice have greater phosphorylation of the myofilament regulatory protein cardiac troponin I (cTnI), which decreases sensitivity of cardiomyocytes to Ca2⁺ and may lead to cardiac dysfunction (3). In contrast, cardiac-specific overexpression of a constitutively active PKCε mutant results in mild concentric hypertrophy, normal left ventricular performance, no change in cTnI phosphorylation, and an increase in myofilament Ca2⁺ sensitivity (9–11). Since both PKCβ and PKCε could phosphorylate cTnI (12), it remains unclear how PKCβII and PKCε can differently regulate cTnI phosphorylation.

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* The abbreviations used are: ROS, reactive oxygen species; PKA, CAMP-dependent protein kinase; PKC, protein kinase C; RSK, ribosomal S6 kinase; p90RSK, p90 ribosomal S6 kinase; cTn, cardiac tropinin I; cTnT, cardiac troponin T; NHE-1, Na+/H⁺ exchanger; ERK, extracellular signal-regulated kinase; PKCα/βII, PKCα/βII transgenic mice; PKCβII, PKCβII transgenic mice; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MOPS, 4-morpholinepropanesulfonic acid; Tg, transgenic; DN, dominant negative.
p90RSK has multiple functions. In quiescent cells, inactive p90RSK resides in the cytoplasm and is partially complexed with its upstream regulator, ERK1/2 (13). Stimulation of the cells by growth factors or ROS operating through Ras/Raf-1/MEK1/2 pathways leads to the activation of ERK1/2, phosphorylation, and activation of p90RSK, and the import of these kinases into nucleus. It has been proposed that p90RSK is involved in activation of nuclear factor-κB by phosphorylation of Ik-B (14) or phosphorolysates transcription factors, including c-Fos (15), Nur77 (16), and cAMP-response element-binding protein (17). Furthermore, we reported that p90RSK is a serum-stimulated Na+/H+ exchanger-1 (NHE-1) kinase and that p90RSK regulates its activity (18). However, the role of p90RSK in the heart remains unclear.

We have previously demonstrated that H$_2$O$_2$-mediated p90RSK activation is partially dependent on PKC activation in Jurkat cells (19). To determine the role of specific PKC isoforms on cardiac p90RSK activation in vivo, we utilized mice with cardiac-specific PKCβII and PKCε overexpression. We show here that p90RSK activation is specifically up-regulated in PKCβII transgenic (Tg) mice but not in PKCε-Tg mice. Since cTnI possesses putative phosphorylation sites of p90RSK at Ser$^{23}$ and Ser$^{24}$, and cTnI phosphorylation only increases in PKCβII-Tg mice, we hypothesized that cTnI (Ser$^{23/24}$) was a p90RSK substrate. We demonstrate here that p90RSK can directly phosphorylate cTnI, and p90RSK activation is required for H$_2$O$_2$-mediated cTnI (Ser$^{23/24}$) phosphorylation. To determine the role of p90RSK activation in vivo, we also generated cardiac-specific p90RSK overexpression mice, and we report here that cTnI (Ser$^{23/24}$) phosphorylation is increased in these mice. Taken together, these data support the importance of p90RSK activation in H$_2$O$_2$-mediated cTnI (Ser$^{23/24}$) phosphorylation.

FIG. 1. Activation of p90RSK and ERK1/2 in PKCβII or PKCe transgenic mice. A, immunoblots (IB) of the PKCβII isofrom in whole cardiac homogenates from NLC and cardiac-specific PKCβII transgenic mice (PKCβII-Tg) (top panel). p90RSK activity was measured by in vitro kinase assay using GST-NHE-1 (amino acids 625-747) as substrate (second panel from the top). No difference in the amount of p90RSK was observed in lysates from any of the heart samples using Western blot analysis with anti-p90RSK (third panel from the top). ERK1/2 activity was measured by Western blot analysis with a phospho-specific ERK1/2 antibody (pERK1/2, second panel from the bottom). No difference in the amount of ERK1 was observed in lysates from any of the heart samples using Western blot analysis with anti-ERK1 (bottom panel). B, densitometric analysis of p90RSK activation in PKCβII-Tg mice. Results were normalized for all experiments by arbitrarily setting the mean densitometry of control normal heart samples to 1.0 (shown in mean ± S.D., n = 3, **, p < 0.01). C, immunoblots of the PKCe isofrom in whole cardiac homogenates from NLC and cardiac-specific PKCe transgenic mice (PKCe-Tg) (top panel). p90RSK activity was measured by in vitro kinase assay using GST-NHE-1 (amino acids 625-747) as substrate (second panel from the top). No difference in the amount of p90RSK was observed in lysates from any of the heart samples using Western blot analysis with anti-p90RSK (third panel from the top). ERK1/2 activity was measured by Western blot analysis with a phospho-specific ERK1/2 antibody (second panel from the bottom). No difference in the amount of ERK1 was observed in lysates from any of the heart samples using Western blot analysis with anti-ERK1 (bottom panel). D, densitometric analysis of p90RSK activation in PKCe-Tg mice. Results were normalized for all experiments by arbitrarily setting the mean densitometry of control normal heart samples to 1.0 (shown in mean ± S.D., n = 3; N.S., not significant).

EXPERIMENTAL PROCEDURES

Protein Extract from Heart Tissue—Mouse hearts were washed with 10 ml of cold phosphate-buffered saline. Isolated heart tissues were frozen in liquid nitrogen and homogenized with 0.5 ml of lysis buffer (10 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.05% Triton X-100, 0.5% sodium deoxycholate, 10% glycerol, 10% β-mercaptoethanol, 100 mM sodium pyrophosphate, 1 μg/ml Aprotinin, 1 mM PMSF, 20 μg/ml Leupeptin, 1 mg/ml PMSF). The tissue lysates were boiled for 5 min in sample buffer (50 mM Tris pH 6.8, 10% glycerol, 8% SDS, 0.01% bromophenol blue) and subjected to electrophoresis on 10% SDS-PAGE gels. The gels were transferred to nitrocellulose membranes, and the membranes were blocked with 5% nonfat milk in TBS-T (50 mM Tris pH 7.4, 0.1 M NaCl, 0.1% Tween 20) for 1 h, followed by overnight incubation with primary antibodies in blocking buffer. The membranes were then incubated with an appropriate secondary antibody conjugated to horseradish peroxidase, and the formation of immune complexes was detected with an enhanced chemiluminescence detection system.

- PKCβ transgenic mice
- PKCe transgenic mice
- Western blot analysis
- Phospho-specific antibodies
- GST-NHE-1 (amino acids 625–747)
- ERK1/2 activity
- Densitometric analysis
- Cardiac-specific PKC isoforms
- cTnI phosphorylation
- H$_2$O$_2$-mediated signaling
- Protein kinase C
- Phospho-specific ERK1/2 antibody
- p90RSK activity
0.05% Nonidet P-40 containing 2 mmol/liter sodium orthovanadate and protease inhibitor mixture (Sigma). Protein concentration was determined with the Bradford protein assay (Bio-Rad). Protein (30 

\textbf{Fig. 2.} \textit{In vitro} phosphorylation of cTnI by p90RSK. \textit{A}, autoradiographs showing specificity for concentration-dependent phosphorylation of TnI and TnT subunits. TnI (up to 10 

\textit{B}, concentration-dependent phosphorylation of cardiac TnI by p90RSK. All phosphorylation reactions were carried out for 15 min in the presence of 5 

\textit{C}, concentration-dependent phosphorylation of cTnI by p90RSK. * indicates degraded products of cTnI.

**p90RSK in Vitro Kinase Assays**—For the autoradiography studies in Fig. 2A, hearts were crushed in liquid nitrogen, homogenized with 3 volumes of lysis buffer, and centrifuged at 14,000 x g (4 °C for 30 min). Protein concentration of supernatants was then determined. p90RSK was immunoprecipitated through the incubation of 1000 

**MALDI-TOF Mass Spectrometry Analysis of cTnI Phosphorylation by p90RSK**—Tryptic digestion of pooled gel slices containing recombinant human cardiac TnI after incubation with recombinant p90RSK was subjected to enzymatic cleavage for the generation of peptide fragments. Pieces were washed with 100 mM ammonium bicarbonate, reduced (dithiothreitol) and alkylated (iodoacetamide), and then dehydrated via acetone evaporation. The gel pieces were reswollen with 25 mM ammonium bicarbonate containing 0.2 

**Western Blot Analysis**—After treatment with reagents or 24 h after adenovirus transduction, the cells were washed with phosphate-buffered saline and harvested in 0.5 ml of lysis buffer as described previously (22). Western blot analysis was performed as described previously (22). In brief, the blots were incubated for 4 h at room temperature with the anti-phospho-cardiac troponin I (Cell Signaling), which recognizes dual phosphorylation of Ser23 and Ser24, anti-troponin I (Cell Signaling), and PKC\textbeta\text{II} activation and anti-ERK1 or 2, p90RSK, and PKC\textalpha/bII activation and anti-ERK1 or 2, p90RSK2, and PKC\textbeta antibody were from Santa Cruz Biotechnology, and the phospho-antibody were from Santa Cruz Biotechnology, and the phospho-

**Adenoavirus Vector Containing Dominant Negative Form of p90RSK**—The dominant negative form of rat p90RSK1 (K93A/K447A) construct was cloned into the Adest28TM-CMV system (QBIOL, Inc.) and transfected into Sf21 insect cells transfected with baculovirus expression vector. Recombinant full-length p90RSK (Upstate Biotechnology), which was purified from Sf21 insect cells transfected with baculovirus expression vector. Reaction mixtures (200 

**AdenoTyro**...
Carlsbad, CA) using Sall and HindIII restriction enzymes as described previously (23).

Preparation of Rat Neonatal Cardiomyocyes—Primary cultures of cardiac myocytes were prepared from ventricles of 1–3-day-old neonatal Wistar rats (24). Briefly, cells were dissociated by collagenase II (Worthington Biochemical) from the ventricles and plated at a density of 1.5 × 10^5 cells/cm² on 35-mm collagen-coated coverslips in culture medium (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and 10% horse serum). After 6 h of plating the isolated cardiomyocytes, 10 μM cytosine 1-β-D-arabinofuranoside was added and cultured 24 h. After that, the culture medium was changed to the medium with 0.5% fetal bovine serum and 10 μM Ara C.

Animals—PKCB transgenic mice were generated by G. L. King as described previously (4), and PKCε transgenic mice were generated by T. Jalili and R. A. Walsh. Briefly, a 2.7-kb fragment containing mouse PKCε cDNA and endogenous polyadenylation sequence (kindly provided by W. J. van Blitterswijk, Netherlands Cancer Institute, Amsterdam, The Netherlands) was subcloned between the 5.5-kb murine α-myosin heavy chain promoter and a 250-bp SV40 polyadenylation sequence (kind gift from J. Robbins, Children’s Hospital Research Foundation, Cincinnati, OH). The purified transgene fragment was injected into male pronuclei of fertilized mouse oocytes (University of Cincinnati Transgenic Core, Cincinnati, OH). Genotype of mouse pups was confirmed by PCR analysis of ear clipping using standard procedures.

Rat p90RSK1 cDNA was subcloned into a pBluescript-based Tg vector between the 5.5-kb murine-α-MHC (α-myosin heavy chain) promoter and the 250-bp SV40 polyadenylation sequences (a kind gift from J. Robbins, Children’s Hospital Research Foundation, Cincinnati, OH). The purified transgene fragment was injected into male pronuclei of fertilized mouse oocytes (University of Rochester Transgenic Core). The genotype of mouse pups was confirmed by PCR analysis of tail clipping using standard procedures.

Materials—We obtained LY333,531 from A.G. Scientific, Inc. (San Diego, CA) and PKCB C2-4 inhibitor from Biomol (Plymouth Meeting, PA).

Statistical Analysis—Values presented are mean ± S.D. Statistical analysis was performed with the StatView 4.0 package (Abacus Concepts). Differences were analyzed with one- or two-way repeated measures analysis of variance as appropriate followed by Scheffe’s correction.

RESULTS

p90RSK Is Activated in PKCBII Transgenic Mice but Not in PKCε Transgenic Mice—It has been reported that cardiomiocyte-specific overexpression of the PKCBII isoform in transgenic mice causes cardiomyopathy, which is characterized by left ventricular hypertrophy, myocardial fibrosis, and decreased in vivo left ventricular performance (4). In these mice, PKCBII-induced phosphorylation of the myofilament regulatory protein cTnI decreases cardiomyocyte calcium sensitivity and may cause depressed cardiomyocyte function (5). On the other hand, the calcium-independent PKCε isoform has been implicated in cardiomiopathy and ischemic preconditioning (25). Of note, unlike PKCBII overexpression mice, transgenic mice with cardiac-specific overexpression of wild type PKCε demonstrated concentric hypertrophy with normal in vivo cardiac function. We evaluated p90RSK activity in both of these transgenic mice and found that p90RSK activation is significantly increased in PKCBII transgenic mice in comparison with control non-transgenic littermate control (NLC) mice (Fig. 1, A and B). However, in PKCε transgenic mice, we could not find any differences in p90RSK activity between NLC and transgenic mice (Fig. 1, C and D). These data are consistent with our hypothesis that p90RSK could contribute to PKCε-mediated depressed cardiac function by phosphorylation of cTnI.

data from three experiments. The initial rate of phosphorylation of human cTnI wild type was studied as shown in Fig. 2B. Since the phosphorylation rate was linear as a function of a 10–20-min incubation time under this experimental condition (data not shown), all phosphorylation reactions were carried out for 15 min in the presence of 5 mM [γ-32P]ATP (2 × 10⁶ cpm). It was found that K_m (μM) = 0.6 ± 0.1, V_max (pmol/min) = 4.6 ± 0.1, and V_max/K_m = 7.5, which were similar to TnI phosphorylation induced by PKCα and PKCδ as reported previously (26). These data support the hypothesis that cTnI is a good substrate for p90RSK.

FIG. 3. p90RSK activation and cTnI (Ser²⁰²⁴) phosphorylation were increased by oxidative stress in cardiomyocytes. A, cardiomyocytes were stimulated for the indicated times with 100 μM H_2O_2. Cells were harvested in lysis buffer, and Western blot analysis was performed with anti-phosphospecific p90RSK antibody (top panel). No difference in the amount of p90RSK was observed in lysates from any of the heart samples using Western blot analysis with anti-p90RSK (second panel from the top). Bottom panel, densitometric analysis of p90RSK activation. Results were normalized to control (time = 0), which was arbitrarily set to 1.0 (shown is mean ± S.D., n = 3). IB, immunoblots. B, cardiomyocytes were stimulated for the indicated times with 100 μM H_2O_2. Cells were harvested in lysis buffer, and Western blot analysis was performed with anti-phosphospecific cTnI (Ser²⁰²⁴) antibody (top panel). No difference in the amount of cTnI was observed in lysates from any of the heart samples using Western blot analysis with anti-cTnI (second panel from the top). Bottom panel, densitometric analysis of cTnI (Ser²⁰²⁴) phosphorylation. Results were normalized to the mean densitometry of control (time = 0), which was arbitrarily set to 1.0 (shown is mean ± S.D., n = 3).
Determination of the Phosphorylation Sites by MALDI-TOF Mass Spectrometry—To determine p90RSK phosphorylation sites on cTnI, we performed tryptic peptide mapping using MALDI-TOF mass spectrometry analysis after recombinant p90RSK was immunoprecipitated and incubated with cTnI in an in vitro kinase reaction. Computer-assisted proteomic analysis revealed two phosphorylated tryptic ions with mass/charge ratios (m/z) of 786.2225 and 942.3236, which correspond to cTnI residues 23–27 + 2PO4 and cTnI residues 22–27 + 2PO4, respectively. Since p90RSK is a Ser/Thr kinase, and the cTnI peptide residues 22–27 contains two Ser at positions 23 and 24, it is most likely that both Ser23 and Ser24 are phosphorylated by p90RSK activation. In addition, we utilized an anti-phosphospecific TnI antibody (Cell Signaling), which recognized dual phosphorylation of Ser23 and Ser24 sites. As shown below (see Fig. 5), we found dual phosphorylation of Ser23/24 in cardiac TnI in p90RSK-Tg mice in vivo and also detected Ser23/24 dual phosphorylation by p90RSK in vitro kinase assay using cTnI as a substrate (data not shown). Taken together, these data suggest that p90RSK phosphorylates cTnI at both Ser23 and Ser24.

Furthermore, we identified four phosphopeptides with m/z 627.2867, 707.2530, 840.4093, and 908.3644, corresponding to 75–79 + 1PO4, 75–79 + 2PO4, 73–79 + 1PO4, and 73–79 + 2PO4, respectively. cTnI peptide residues 73–79 contain Ser at position 78 and Thr at position 79. Since Ser78 contains p90RSK, a part of consensus phosphorylation site (RXXS), Ser78 may be one of the phosphorylation sites by p90RSK. Interestingly, we also found that the phosphopeptides with m/z 693.2374, 821.3324, and 908.3644 correspond to cTnI residues 41–45 + 2PO4, 41–46 + 2PO4, and 39–45 + 2PO4, respectively. Of note, cTnI 41–45 residues contain reported PKC phosphorylation sites of Ser42 and Ser44 in human cTnI (12). These data suggest that p90RSK can phosphorylate cTnI at multiple sites.

H2O2 Induced cTnI (Ser23/24) Phosphorylation via p90RSK Activation in Cardiomyocytes—ROS and growth factors stimulate similar intracellular signal transduction events including activation of Src kinase family members and extracellular signal-regulated kinase (ERK1/2) (27–29). To determine whether ROS activates both p90RSK and cTnI phosphorylation, we used H2O2 (100 μM) to stimulate p90RSK activity and cTnI phosphorylation. Of note, we investigated here the Ser23/24 (20RRRSS24) phosphorylation of cTnI using anti-dual phosphospecific (Ser23/24) TnI antibody, which have been reported as PKA phosphorylation sites because these sites have a significant role in regulating TnI function (12) and contain putative consensus sequence of p90RSK (RRXXS). As shown in Fig. 3A, we found that H2O2 stimulated p90RSK activity after 10 min and was sustained up to 60 min. Using an anti-dual phosphospecific Ser23/24 cTnI antibody, we found that cTnI phosphorylation was increased after 10 min and also sustained after 60 min of stimulation, paralleling the time course of H2O2-mediated p90RSK activation.

To determine the role of p90RSK activation in H2O2-mediated cTnI phosphorylation, we generated an adenovirus vector...
containing the dominant negative form of p90RSK (Ad-DN-p90RSK) and transfected it into cardiomyocytes. As shown in Fig. 4, A (second panel from the top) and B (upper panel), we found that H$_2$O$_2$-mediated cTnI phosphorylation was significantly inhibited in Ad-DN-p90RSK transfected cells. In contrast, we did not observe any inhibition on ERK1/2 activation (Fig. 4, A, second panel from the bottom, and B, lower panel). We also confirmed that p90RSK activity was significantly decreased in Ad-DN-90RSK transfected cells (Fig. 4C). These data suggest that p90RSK activation is critical for H$_2$O$_2$-mediated cTnI phosphorylation.

**cTnI Phosphorylation in Cardiac-specific p90RSK Transgenic Mice (p90RSK-Tg)—**To examine the effect of activation of p90RSK on cTnI phosphorylation at the whole organ level, we created Tg mice with cardiac-specific expression of the wild type of p90RSK. The level of Tg protein expression in three different lines of Tg mice was determined by Western blot using an anti-p90RSK antibody. We found an 8–9-fold increase in the level of p90RSK expression in all Tg lines relative to wild type mice, and there was a concordant 10–12-fold increase in p90RSK phosphorylation. However, there was no significant ERK1/2 and PKCα/βII activation in p90RSK-Tg mice, confirming a selective activation of p90RSK (Fig. 5). cTnI (Ser23/24) phosphorylation was significantly increased in p90RSK-Tg mice (Fig. 5, A, third panel from the top, and B, upper panel), which was also observed in other two lines of p90RSK-Tg mice (data not shown). These data support a role for p90RSK activation in cTnI (Ser23/24) phosphorylation in vivo.

**Role of PKCβ in H$_2$O$_2$-mediated p90RSK Activation and cTnI (Ser23/24) Phosphorylation in Cardiomyocytes—**It has been reported that Ser43/45 and Thr414 of cTnI are phosphorylation sites of PKC, but it is unclear whether PKC activation can indirectly result in greater Ser23/24 phosphorylation because only minor phosphorylation of Ser23/24 has been observed by PKC (9). Since we found that p90RSK activation was increased in PKCβII-Tg mice and that p90RSK could directly phosphorylate cTnI in vitro, we investigated whether PKCβ can regulate H$_2$O$_2$-mediated p90RSK activation and subsequent dual TnI (Ser23/24) phosphorylation in cardiomyocytes. To determine the role of PKCβ activation in H$_2$O$_2$-mediated p90RSK activation and cTnI phosphorylation, we used a myristoylated PKCβ II inhibitor that inhibits the translocation and function of all classical PKC isoforms (30, 31) and PKCβ-specific inhibitor, LY333,531. We confirmed that LY333,531 did not directly inhibit p90RSK activation by directly adding LY333,531 (20, 200 nM) in p90RSK in vitro kinase assay mixture (data not shown). As shown in Fig. 6, pretreatment with PKCβ C2-4 inhibitor and LY333,531 abolished H$_2$O$_2$-mediated p90RSK activation (Fig. 6, A and C, top panel, and B and D, upper panel) and cTnI (Ser23/24) phosphorylation (Fig. 6, A and C, third panel from the top, and B and D, lower panel). These data indicate that PKCβ activation is important for p90RSK activation as well as downstream cTnI (Ser23/24) phosphorylation.

**DISCUSSION**

In the present study, we found that p90RSK activation regulates H$_2$O$_2$-mediated cTnI (Ser23/24) phosphorylation. To our knowledge, this is the first report to document cTnI (Ser23/24) phosphorylation by p90RSK. In addition, we found that p90RSK activation and cTnI (Ser23/24) phosphorylation were increased in PKCβII-Tg but not in PKCε-Tg mice and that the PKCβ inhibitor prevented H$_2$O$_2$-mediated p90RSK activation and cTnI (Ser23/24) phosphorylation. These data support a critical role for PKCβII in regulating p90RSK activation and subsequent cTnI (Ser23/24) phosphorylation. It has been reported that specific PKC-mediated phosphorylation of Ser43/45 of cTnI plays an important role in regulating force development in cardiac muscle (9). Since p90RSK kinase, which is downstream of PKCβII, could phosphorylate Ser23/24 in N-terminal of cTnI, and PKC phosphorylation sites of Ser43/45 already exist on cTnI, there appears to be a novel complexity of PKCβII activation on cTnI function in vivo.

p90RSK consists of three isoforms that show the same overall structure consisting of two kinase domains, a linker region, and short N-terminal and C-terminal tails. The C-terminal kinase belongs to the calcium/calmodulin-dependent kinase group of kinases, but the only known function of the C-terminal kinase is the regulation of the activity of N-terminal kinases. The N-terminal kinase belongs to the AGC group of kinases, which include PKA and PKC. The N-terminal kinase is responsible for phosphorylating the RSK substrates and recognizes the basic consensus motif: (R/K)(S/T), or RR(S/T), which fits to both Ser23 and Ser44 of cTnI (25) (32, 33).

In the current study, we also determined that human cardiac TnI residues 41–45 can be phosphorylated by p90RSK using MALDI-TOF mass spectrometry. TnI residues 41–45 contains two Ser at positions 42 and 44, which have been reported as major PKC-dependent phosphorylation sites in cardiac TnI (12). Although these sites do not show a consensus sequence of p90RSK phosphorylation sites, it is still possible that p90RSK can phosphorylate these Ser42 and Ser44 sites because the RSK amino-terminal kinase domain is homologous to the kinase domains in other members of the AGC family, including PKA, various PKCs, and p70RSK (34).

It should also be mentioned that during the preparation of this study, Kobayashi et al. (35) have published that Ser23 or Ser44 of cTnI is the most permissive site for PKC-dependent
phosphorylation, and the phosphorylated cTnI with Ser 42 and/or Ser44, previously reported as the phosphorylation sites of PKC (12), could not be detected. They explained the discrepancy of their data with a previous report (12) by the different usage of their recombinant PKC (Kobayashi et al. (35)) instead of a mixture of PKC isoforms from brain (Noland et al. (12)). Since we found that endogenous p90RSK activation was regulated by PKC/II (Fig. 6) and also detected coimmunoprecipitation of endogenous p90RSK with PKC/II (data not shown), it may be possible that activation of p90RSK is responsible for Ser42/44 phosphorylation in a mixture of PKC isoforms from brain. Further investigation is necessary to clarify this issue.

The activation of PKC and increased diacylglycerol levels initiated by hyperglycemia are associated with many vascular abnormalities in retinal, renal, and cardiovascular disease (36, 37). It has been reported that increased PKC activity in diabetic rat hearts could be due to β and ζ isoforms (36). Although we could not detect ERK1/2 activation in PKCβ-Tg mouse hearts, we observed significant activation of p90RSK. We have previously reported the presence of another ROS-stimulated pathway that activates p90RSK independently of MEK1/2 and ERK1/2 (19). It is possible that PKCβII directly phosphorylates p90RSK and activates p90RSK activation, but this is beyond the scope of this study.

Desensitization of myofibrils to Ca²⁺ appears to oppose the inotropic effect produced by phospholamban and sarcolemmal L-type calcium channel phosphorylation, but several studies also indicate that cTnI phosphorylation by PKA plays an essential role in the rate of force production (10, 11, 38). PKA phosphorylation of cTnI increases the rates of cross-bridge cycling, and transgenic mouse hearts expressing constitutively phosphorylated cTnI at PKA phosphorylation sites exhibit augmented force production and faster relaxation (39). We generated cardiac-specific p90RSK transgenic mice and found that TnI (Ser23/24) phosphorylation was increased as shown in Fig. 5. In the current study, we focused on the role of p90RSK to phosphorylate cTnI (Ser 23/24), as a downstream event to PKCβII activation.

![Fig. 6. PKCβ C2-4 inhibitor and PKCβ-specific inhibitor, LY333,531, prevented H₂O₂-mediated p90RSK activation and cTnI (Ser²³/²⁴) phosphorylation in cardiomyocytes.](image-url)

A. Cardiomyocytes were treated with myristoylated PKCβ C2-4 inhibitor (10 μM) (A) and LY333,531 (200 nM) (B) followed by H₂O₂ stimulation for 20 min at 100 μM. p90RSK phosphorylation was detected by Western blotting with anti-phosphospecific p90RSK antibody (top panel). cTnI (Ser²³/²⁴) phosphorylation was measured by Western blot analysis with a phospho-specific cTnI (Ser²³/²⁴) antibody (p-TnI, third panel from the top). No difference in the amount of p90RSK (second panel from the top) and cTnI (bottom panel) was observed in lysates from any of the heart samples using Western blot analysis with anti-p90RSK and TnI antibodies. B and D, densitometric analysis of p90RSK activation (upper) and cTnI (Ser²³/²⁴) phosphorylation (lower) with or without PKCβ C2-4 inhibitor (B) or LY333,531 (D) treatment. Results were normalized to the mean densitometry of control (time = 0) from vehicle-treated cells, which was arbitrarily set to 1.0 (shown is mean ± S.D., n = 3).
clarify the role of p90RSK activation on cardiac function and Ca\textsuperscript{2+} sensitivity via cTnI (Ser\textsuperscript{23/24}) phosphorylation.

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