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A novel protein kinase C target site in protein kinase D is phosphorylated in response to signals for cardiac hypertrophy

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ABSTRACT

Protein kinase D (PKD) regulates cardiac myocyte growth and contractility through phosphorylation of proteins such as class IIa histone deacetylases (HDACs) and troponin I (TnI). In response to agonists that activate G-protein-coupled receptors (GPCRs), PKD is phosphorylated by protein kinase C (PKC) on two serine residues (Ser-738 and Ser-742 in human PKD1) within an activation loop of the catalytic domain, resulting in stimulation of PKD activity. Here, we identify a novel PKC target site located adjacent to the auto-inhibitory pleckstrin homology (PH) domain in PKD. This site (Ser-412 in human PKD1) is conserved in each of the three PKD family members and is efficiently phosphorylated by multiple PKC isozymes *in vitro*. Employing a novel anti-phospho-Ser-412-specific antibody, we demonstrate that this site in PKD is rapidly phosphorylated in primary cardiac myocytes exposed to hypertrophic agonists, including norepinephrine (NE) and endothelin-1 (ET-1). Differential sensitivity of this event to pharmacological inhibitors of PKC, and data from *in vitro* enzymatic assays, suggest a predominant role for PKCδ in the control of PKD Ser-412 phosphorylation. Together, these data suggest a novel, signal-dependent mechanism for controlling PKD function in cardiac myocytes.

1. Introduction

Biomechanical stress triggers cardiac hypertrophy, in part, through activation of autocrine/paracrine signaling pathways that stimulate Gαq/Gα11 protein-coupled receptors (GPCRs), including the angiotensin, endothelin and α_1-adrenergic receptors. Mice with compound knockout of the genes encoding Gαq and Gα11, which are functionally redundant, are resistant to pressure overload-induced cardiac hypertrophy [37]. Cardiac hypertrophy has long been viewed as a compensatory mechanism that normalizes wall stress and enhances cardiac performance. However, long-term suppression of cardiac hypertrophy is associated with reduced morbidity and mortality in patients with hypertension, and thus chronic cardiac hypertrophy is considered maladaptive [4,9]. Furthermore, studies in animal models have demonstrated that blocking "compensatory" hypertrophy can result in improved cardiac function and provide long-term survival benefit [8]. As such, there is interest in further elucidating signaling mechanisms that control cardiac growth.

Among the downstream effectors of Gαq-coupled receptors in cardiac myocytes are members of the protein kinase D (PKD) family [23]. The three PKD isoforms are highly homologous, consisting of ~900 amino acids, with two amino-terminal cysteine-rich domains (CRDs) that mediate binding to diacylglycerol (DAG), an internal pleckstrin homology domain (PH) and carboxy-terminal catalytic domains [29]. The CRD and PH domains appear to serve auto-inhibitory functions [16,17]. PKD is activated in a manner largely dependent on phosphorylation of activation loop sites in the catalytic domain by "novel", calcium-independent protein kinase C isozymes (nPKCs) [18,26,28,36,38], although PKC-independent auto-phosphorylation of these sites has also been described [19,30,35]. *In vivo*, cardiac-specific expression of constitutively active PKD1 in mice causes a brief phase of cardiac hypertrophy, followed by chamber dilation and impaired systolic function [34]. Mice in which PKD1 was specifically deleted in cardiac myocytes showed dramatically reduced cardiac hypertrophy

Abbreviations: CHK, checkpoint kinase; CRD, cysteine rich domain; DAG, diacylglycerol; ET-1, endothelin-1; GPCR, G-protein-coupled receptor; GSK-3β, glycogen synthase kinase-3β; HDAC, histone deacetylase; IKK, IκB kinase; ISO, isoproterenol; MARK, microtubule associated kinase; MyBP-C, myosin binding protein-C; NE, norepinephrine; nPKC, novel protein kinase C; NRVM, neonatal rat ventricular myocyte; PE, phenylephrine; PH, pleckstrin homology; PKC, protein kinase C; PKD, protein kinase D; PMA, phorbol myristate acetate; TnI, troponin I.

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and cardiac fibrosis in response to pressure overload or chronic administration of adrenergic or angiotensin receptor agonists [7].

We performed a series of biochemical studies to further elucidate mechanisms of PKD regulation in cardiac myocytes. A novel, signal-responsive phosphorylation site residing adjacent to the PH domain in PKD was discovered. This phospho-acceptor site is targeted by nPKC family members, including PKCδ. The data suggest an expanded role for PKC in the control of PKD in cardiac myocytes.

2. Materials and methods

2.1. Reagents and DNA constructs

Phorbol 12-myristate 13-acetate (PMA), isoproterenol (ISO), endothelin-1 (ET-1), phenylephrine (PE), and Norepinephrine (NE) were purchased from Sigma. Gö6976 and Gö6983 were obtained from Calbiochem. PKD1 deletion constructs were generated by PCR using Pfx polymerase (Invitrogen) and cloned into the pcDNA3.1 mammalian expression vector (Invitrogen). Expressed proteins harbored an amino-terminal Myc tag. Site directed mutagenesis was performed using a QuickChange kit (Stratagene). The following antibodies were purchased: anti-Myc (Santa Cruz Biotechnology; sc-40 and sc-788), anti-calnexin (Santa Cruz Biotechnology; sc-11397), anti-PKD1 (Cell Signaling Technology; #2052), anti-phospho-PKD1 Ser-738/742 (Cell Signaling Technology; #2054), anti-phospho-PKD1 Ser-910 (Cell Signaling Technology; #2051) anti-PKD substrates antibody (Cell Signaling Technology; #4381). Anti-phospho-PKD1 Ser-412 was custom made by Invitrogen using a peptide antigen based on amino acids 404–416 of human PKD1 (Ile-Pro-Leu-Met-Arg-Val-Val-Gln-Ser_{412}(P)-Val-Lys-His-Thr). Rabbit antiserum was affinity purified using the peptide antigen and specificity of the antibody for phospho-PKD was confirmed by ELISA.

2.2. Cell culture and transient transfection

HEK293T cells were maintained in Dulbecco's mininimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and penicillin–streptomycin. Cells were transiently transfected with 1 μg of the indicated PKD plasmid constructs using Fugene 6 (Roche Molecular Biochemicals). Sixteen hours post-transfection, cells were starved in serum-free medium supplemented with 0.1% of Neutridoma-SP (0.1%; Roche Applied Sciences) for 3 h and subsequently stimulated with PMA for 1 h. Neonatal rat ventricular myocytes (NRVMs) were prepared from 1 to 2 day old Spraque–Dawley rats as described previously [12]. NRVMs were cultured on 0.1% gelatin-coated plates overnight, starved for 3 h as described for 293T cells and treated with agonists for 1 h. Adenovirus was used at a multiplicity-of-infection of 50.

2.3. Immunoprecipitation and immunoblotting

HEK293T cells or NRVMs were harvested in lysis buffer containing Tris (50 mM, pH 7.5), EDTA (5 mM), Triton X-100 (1%), protease inhibitor cocktail (Roche), phenylmethylsulfonyl fluoride (1 mM), sodium pyrophosphate (1 mM), sodium fluoride (2 mM), β-glycerol phosphate (10 mM), sodium molybdate (1 mM), and sodium orthovanadate (1 mM). Lysates were sonicated for 10 s and cell debris was removed by centrifugation. For immunoprecipitation, cell lysates (500 μl; ~250 μg total protein) were mixed with monclonal anti-Myc antibody (1 μg) for 1 h at 4 °C followed by addition of protein G agarose beads (20 μl packed volume; GE Biosciences) and incubated overnight. Beads were washed three times with lysis buffer and bound proteins were resolved by SDS–PAGE and transferred to nitrocellulose membranes. Proteins were detected using a chemiluminescence system (Pierce).

2.4. In vitro kinase assays

Kinase activity was measured using a biotinylated PKD Ser-412 peptide [Ile-Pro-Leu-Met-Arg-Val-Val-Gln-Ser_{412}-Val-Lys-His-Thr] (American Peptide Company). All recombinant kinases were purchase from Millipore. Standard reaction mixtures contained 20 μM biotinylated peptide, 20 μM [γ-^{32}P] ATP (~1×10^5 cpm; Perkin–Elmer), 1 mM DTT, 10 mM $MgCl_2$, 50 mM Hepes buffer, pH. 7.6 and 25 ng of the indicated kinases (Upstate Biotechnolog) in a final volume of 50 μl. For PKCα activity, 0.5 μg of lipid activator complex containing DAG and phosphatidylserine (Millipore) and 1 mM of $CaCl_2$ were added to the reaction mixture. After incubation at 25 °C for 30 min, 10 μl of each reaction mixture was withdrawn and added to streptavidin-coated 96-well plates (Promega). Wells were washed sequentially with 1.2 ml of 2 M NaCl, 1.2 ml of 2 M NaCl containing 1% H_3PO_4, 600 μl of H_2O and 200 μl 95% ethanol. Plates were allowed to dry at 25 °C for 1 h and then 25 μl of scintillation fluid (Microscint-20 from Perkin–Elmer) was added to each well. Incorporation of [γ-^{32}P] ATP into the peptide substrate was measured using a Top-Count NXT (Packard).

3. Results and discussion

In the heart, PKD has been shown to phosphorylate class IIa HDACs [34], cardiac troponin I (cTnI) [2,10,14], myosin binding protein C (MyBP-C) [1] and CREB [11,25]. To further address the role of PKD in the heart, experiments were performed to identify other putative substrates of this kinase. PKD is potently activated in cardiac myocytes in response to norepinephrine (NE)-mediated stimulation of α_1-adrenergic receptors [15]. Rats were treated with NE for 1 h and left ventricular (LV) protein homogenates were prepared. LV proteins were resolved by SDS–PAGE and analyzed by immunoblotting with the anti-PKD substrates antibody [6], which was developed against a synthetic peptide based on the optimal PKD substrate sequence [24] (Fig. 1A). As shown in Fig. 1B, NE dramatically enhanced phosphorylation of cardiac proteins that were recognized by this antibody.

A prominent signal was observed near the 120 kDa marker. Additional immunoblotting revealed that PKD1 from unstimulated and NE-stimulated cardiomyocytes co-migrated with this protein (Fig. 1C), suggesting the possibility that the PKD substrates antibody recognizes an auto-phosphorylated form of PKD1. To begin to address this possibility, PKD1 was overexpressed in cultured neonatal rat ventricular myocytes (NRVMs) using an adenovirus vector. Cells were stimulated with NE or the DAG analog, PMA, which is a potent inducer of PKC-mediated PKD activation. As shown in Fig. 1D, both agonists triggered auto-phosphorylation of ectopically expressed PKD1, as evidenced by immunoblotting with an antibody that recognizes the canonical auto-phosphorylation site at Ser-910 in human PKD1 (upper panel). Ectopic PKD1 from stimulated cells was also recognized by the PKD substrates antibody (middle panel). However, the antibody failed to recognize recombinant PKD1 that had been produced in insect cells and auto-phosphorylated *in vitro* (Fig. 1E). Together, these results suggest that the PKD substrates antibody recognizes a phosphorylation site in PKD1 that is distinct from the auto-phosphorylation site at Ser-910.

A series of PKD1 deletion constructs was prepared in order to map the phosphorylation site(s) in the kinase that are recognized by the PKD substrates antibody (Fig. 2A). The initial group of truncation mutants was prepared to progressively remove ~100 amino acids from the carboxyl-terminus of PKD1. HEK293T cells