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β-Adrenergic regulation of cardiac type 2A protein phosphatase through phosphorylation of regulatory subunit B56δ at S573

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ABSTRACT

Background: Type 2A protein phosphatase (PP2A) enzymes are serine/threonine phosphatases which comprise a scaffold A subunit, a regulatory B subunit and a catalytic C subunit, and have been implicated in the dephosphorylation of multiple cardiac phosphoproteins. B subunits determine subcellular targeting, substrate specificity and catalytic activity, and can themselves be regulated by post-translational modifications. We explored potential β-adrenergic regulation of PP2A in cardiomyocytes through phosphorylation of the regulatory B subunit isoform B56δ.

Methods and results: Phosphate affinity SDS-PAGE and immunoblot analysis revealed increased phosphorylation of B56δ in adult rat ventricular myocytes (ARVM) exposed to the β-adrenergic receptor (βAR) agonist isoprenaline (ISO). Phosphorylation of B56δ occurred at S573, primarily through stimulation of the β_1AR subtype, and was dependent on PKA activity. The functional role of the phosphorylation was explored in ARVM transduced with adenoviruses expressing wild type (WT) or non-phosphorylatable (S573A) B56δ, fused to GFP at the N-terminus. C subunit expression was increased in ARVM expressing GFP-B56δ-WT or GFP-B56δ-S573A, both of which co-immunoprecipitated with endogenous C and A subunits. PP2A activity in cell lysates was increased in response to ISO in ARVM expressing GFP-B56δ-WT but not GFP-B56δ-S573A. Immunoblot analysis of the phosphoproteome in ARVM expressing GFP-B56δ-WT or GFP-B56δ-S573A with antibodies detecting (i) phospho-serine/threonine residues in distinct kinase substrate motifs or (ii) specific phosphorylated residues of functional importance in selected proteins revealed a comparable phosphorylation profile in the absence or presence of ISO stimulation.

Conclusions: In cardiomyocytes, βAR stimulation induces PKA-mediated phosphorylation of the PP2A regulatory subunit isoform B56δ at S573, which increases associated PP2A catalytic activity. This is likely to regulate the phosphorylation status of specific B56δ-PP2A substrates, which remain to be identified.

1. Introduction

Type 2A protein phosphatase (PP2A) holoenzymes are present in most cell types, including cardiac myocytes, where they dephosphorylate phospho-serine (Ser, S) and phospho-threonine (Thr, T) residues in proteins. PP2A holoenzymes comprise a 65-kDa scaffold ($PP2A_A$ or A) subunit, a 36-kDa catalytic ($PP2A_C$ or C) subunit and a regulatory ($PP2A_B$ or B) subunit of variable molecular weight that determines subcellular targeting, substrate specificity and catalytic activity [1–4]. The importance of B subunits and regulated catalytic activity in the heart is highlighted by the dilated cardiomyopathy phenotype of mice expressing a mutant A subunit that binds the C but not the B subunit [5], and by the impaired cardiac function of mice with cardiomyocyte-specific overexpression of the C subunit [6].

B subunits are classified into three families, PR55/B, PR61/B′ and PR72/B″. The largest of these is the PR61/B′ family, which is commonly referred to as the B56 family and comprises α, β, γ, δ and ε isoforms [2]. Conserved sequences residing in the central domain of B56 isoforms enable association with the AC dimer, whilst unique sequences at the N- and C-terminals determine isoform-specific functions [7,8]. Regarding the role of individual isoforms in the heart in vivo, studies in mice with global deletion [9] or cardiac-specific overexpression [10] of B56α

Abbreviations: ARVM, adult rat ventricular myocytes; CaMKII, calcium/calmodulin-dependent kinase II; cMyBP-C, cardiac myosin binding protein-C; cTnI, cardiac troponin I; I-1, inhibitor of protein phosphatase-1; ISO, isoprenaline; KO, knock out; MOI, multiplicity of infection; BNZ, N^6-benzoyl-cAMP; PLB, phospholamban; PKA, protein kinase A; Ser, S, serine; Thr, T, threonine; PP1, type 1 phosphatase; PP2A, type 2A protein phosphatase; WT, wild type; βAR, β-Adrenergic receptor

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indicate that this B subunit regulates contractility, potentially through phospho-regulation of proteins involved in excitation-contraction (EC) coupling. Studies in mice with global deletion of B56γ indicate that this isoform regulates cardiac development [11].

The focus of this study is B56δ. The gene encoding B56δ in humans (PPP2R5D) is localized in chromosome region 6p21.1 and gives rise to three splice variants that differ at the N-terminus [12–14]. With 602 amino acids and a predicted molecular weight of 70-kDa, δ1 is the largest splice variant. Relative to this splice variant, δ2 and δ3 lack amino acids 84-115 and 1-115, respectively. Studies in vitro have shown that the activity of B56δ-PP2A can be increased by protein kinase A (PKA), through phosphorylation of B56δ at S60, S75, S88 and S573 [15–17]. In non-cardiac cells, B56δ phosphorylation at S573 is necessary for increased B56δ-PP2A activity and is induced by G protein-coupled receptors that signal via the Gs-adenylate cyclase-cAMP-PKA pathway such as dopamine D1 and luteinizing hormone receptors [16,18].

Little information is available regarding B56δ expression, regulation and function in the heart. Mice lacking B56δ were the first mice with targeted disruption of a B56 subunit to be studied; however, their cardiac phenotype was not studied [19]. More recently, DeGrande et al. [20] have shown that B56δ protein is expressed in mammalian heart chambers, and that its levels are increased in dog hearts with ischemic or non-ischemic heart failure.

In view of the importance of the Gs-adenylate cyclase-cAMP-PKA pathway in mediating cardiomyocyte responses to β-adrenergic receptor (βAR) stimulation [21,22], we have explored potential β-AR-mediated regulation and role of B56δ phosphorylation in adult rat ventricular myocytes (ARVM). We have found that (i) B56δ is phosphorylated at S573 following the acute stimulation of βARs, (ii) this response occurs primarily downstream of the β_1AR and is mediated by PKA, and (iii) B56δ phosphorylation at S573 is necessary for βAR-mediated stimulation of PP2A activity.

2. Materials and methods

2.1. Materials

cTnI, pS23/24 cTnI, $PP2A_C$, PLB and phospho-S/T kinase substrate antibodies were from Cell Signaling Technology (2002, 4002, 2038, 8495 and 9920, respectively). GAPDH, H2B and B56γ antibodies were from Abcam (Ab9482, Ab1790-100 and Ab94633, respectively). B56α and B56δ antibodies were from BD Biosciences (610615) and Bethyl (A301-100A), respectively. $PP2A_A$ and B55α antibodies were from Santa Cruz (sc-74580 and sc-81606, respectively). GFP and α-Actinin antibodies were from Roche (11814460001) and Sigma (A7732), respectively. pS16 PLB and pS282 cMyBP-C antibodies were from Badrilla (A010-12AP) and Enzo Life Sciences (ALX-215-057-R050), respectively. The cMyBP-C antibody was a kind gift from Professor Mathias Gautel [23]. pS273 and pS302 cMyBP-C antibodies were a kind gift from Dr. Jeffrey Robbins [24,25]. The pS573 B56δ antibody was a kind gift from Professor Angus Nairn [16]. Cy3- and Cy5-conjugated secondary antibodies were from Jackson ImmunoResearch (115165146 and 111175144, respectively). Isoprenaline (ISO), propranolol (PRO), CGP-20712A (CGP) and ICI 118,551 (ICI) were from Sigma (I5627, P0084, C231 and I127, respectively). H89, myristoylated PKA inhibitor 14-22 amide (PKI), N^6-benzoyl cAMP (BNZ), and okadaic acid were from Calbiochem (371962, 476485, 116802 and 495609, respectively). The pEGFP-C1 vector encoding *PPP2R5D* and cardiac tissue from littermate B56δ knock out (KO) and WT mice were kind gifts from Professor Veerle Janssens [19]. Adult male Wistar rats (300–324 g) were from Harlan Laboratories (UK).

2.2. Construction of adenoviral vectors

To replace S573 with alanine, a single point mutation was introduced into human *PPP2R5D* in a pEGFP-C1 vector using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). The adenoviral vectors expressing wild type (WT) B56δ (AdV.GFP-B56δ-WT) and mutated (S573A) B56δ (AdV.GFP-B56δ-S573A) were constructed using the AdEasy system [26]. In brief, GFP-B56δ cDNA was subcloned into pShuttle-CMV (Stratagene) and homologous recombination of this with pAdEasy-1 (Stratagene) was performed in bacterial cells. Adenoviruses were amplified in HEK293 cells and purified using a cesium chloride density gradient in combination with ultracentrifugation. The infectious titer of the purified adenoviruses was determined in tissue culture infectivity dose 50 assays [27].

2.3. Isolation, culture and adenoviral transduction of ARVM

ARVM were isolated from the hearts of adult male Wistar rats by collagenase-based enzymatic digestion, as previously described [28,29]. Isolated cells were resuspended in Hank's M199 medium supplemented with 2 mM L-creatine, 5 mM carnitine, 5 mM taurine and 100 IU/ml penicillin/streptomycin, and were cultured in plastic 6-well plates pre-coated with laminin. Cells were maintained in a humidified incubator (5% CO_2, 37 °C) for 2 h after which, the medium was replaced with fresh medium and cells were incubated overnight. Where indicated, cells were transduced with adenoviruses 2 h post-plating. AdV.GFP was used at MOI 30. AdV.GFP-B56δ-WT and AdV.GFP-B56δ-S573A were both used at MOI 100.

2.4. Pharmacological treatment of ARVM

Unless otherwise stated, ARVM were incubated with vehicle or 10 nM ISO for 10 min. PRO (100 nM), CGP (100 nM), ICI (100 nM) or vehicle was added to the culture medium 10 min before ISO stimulation. H89 (10 μM), PKI (10 μM) or vehicle was added 30 min before ISO stimulation. Cells were exposed to BNZ (500 μM) or vehicle for 30 min, and to OA (0.1 or 1 μM) or vehicle for 60 min. Experiments were performed at 37 °C.

2.5. Subcellular fractionation of ARVM

The subcellular fractionation method was adapted from methods described in previous publications [29,30]. In brief, cells were harvested in ice-cold lysis buffer containing: 50 mM Tris (pH 7.5), 5 mM EGTA, 2 mM EDTA, 100 mM NaF, 1% (v/v) Triton-X100 and complete mini protease inhibitor (Roche). Cell lysates were incubated on ice for 5 min after which, they were centrifuged at 14,000*g* for 30 min at 4 °C. Proteins in the soluble fraction (supernatant) were denatured in 3X Laemmli sample buffer. Proteins in the insoluble fraction (pellet) were resuspended in 1X Laemmli sample buffer.

2.6. SDS-PAGE and immunoblot analysis

Heat-denatured protein samples were resolved on Tris-glycine SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked in Tris-buffered saline with 0.1% Tween-20 (TBST) and 5% (w/v) non-fat milk. Incubation with primary antibodies was performed overnight at 4 °C and incubation with secondary antibodies was performed for 2 h at room temperature. Protein bands were visualized on chemiluminescence film following incubation of the membranes with ECL Western Blotting Detection Reagents (GE Healthcare). Signal intensity was quantified on a calibrated GS-800 densitometer (Bio-Rad). Phosphate affinity (PhosTag) SDS-PAGE was performed on Tris-glycine gels containing 50 μM acrylamide-pendent PhosTag™ and 100 μM $MnCl_2$. Prior to transfer of proteins to PVDF membranes, gels were incubated 15 min in transfer buffer containing 1 mM EDTA then 15 min in transfer buffer alone.