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Pharmacological inhibition of β IIPKC is cardioprotective in late-stage hypertrophy

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Abstract

We previously found that in the hearts of hypertensive Dahl salt-sensitive rats, β IIPKC levels increase during the transition from compensated cardiac hypertrophy to cardiac dysfunction. Here we showed that a six-week treatment of these hypertensive rats with a β IIPKC-specific inhibitor, β IIV5-3, prolonged their survival by at least six weeks, suppressed myocardial fibrosis and inflammation, and delayed the transition from compensated hypertrophy to cardiac dysfunction. In addition, changes in the levels of the Ca^{2+} -handling proteins, SERCA2 and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, as well as troponin I phosphorylation, seen in the control-treated hypertensive rats were not observed in the β IIPKC-treated rats, suggesting that β IIPKC contributes to the regulation of calcium levels in the myocardium. In contrast, treatment with the selective inhibitor of β IPKC, an alternative spliced form of β IIPKC, had no beneficial effects in these rats. We also found that β IIV5-3, but not β IV5-3, improved calcium handling in isolated rat cardiomyocytes and enhanced contractility in isolated rat hearts. In conclusion, our data using an *in vivo* model of cardiac dysfunction (late-phase hypertrophy), suggest that β IIPKC contributes to the pathology associated with heart failure and thus an inhibitor of β IIPKC may be a potential treatment for this disease.

1. Introduction

Despite the advances in pharmacological interventions, improvements in cardiac devices and in heart transplantation, mortality associated with heart failure continues to increase [1]. Therefore, the identification of novel therapeutic targets for the treatment of heart failure remains a major priority. Because there was an increase in β protein kinase C (β PKC) levels in failing human hearts [2, 3] and in a rat model of hypertension-induced cardiac dysfunction by high-salt diet [4], we set out to determine the role of β PKC isozyms, β I and β IIPKC in cardiac dysfunction model using Dahl rats fed high-salt diet.

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Conflict of interest statement

DM-R is the founder of KAI Pharmaceuticals, Inc, a company that plans to bring PKC regulators to the clinic. However, none of the work described in this study is based on or supported by the company. Other authors have no disclosure.

We used isozyme-selective β I- and β IIPKC inhibitors, β IV5-3 and β IIV5-3, which were previously developed in our lab [5-7]. These six amino acid peptide inhibitors were derived from the least homologous sequence in the only divergent region in these alternatively spliced forms of β PKC[5]. These isozyme-specific peptide inhibitors are linked to membrane permeable peptides, TAT₄₇₋₅₇ [8], to enable their effective delivery into cells and make them useful pharmacological tools. We tested here the possibility that selective pharmacological inhibition of β I- or β IIPKC could inhibit the progression of cardiac dysfunction in these hypertensive rats.

2. Materials and Methods

2.1. Peptide synthesis

β IV5-3 (β IPKC inhibitor, corresponding to amino acids 646-651 [KLFIMN]) and β IIV5-3 (β IIPKC inhibitor, amino acids 645-650 [QEVIRN] [5], were synthesized and conjugated to TAT carrier peptide (amino acids 47-57 [YGRKKRRQRRR]) *via* a disulfide bond between Cys residues at the N-terminus of each peptide [9] by American Peptide, Inc. (Sunnyvale, CA).

2.2 Hypertension-induced rat model of cardiac dysfunction

Male Dahl rats were obtained from Harlan Sprague-Dawley (Indianapolis, Indiana). Rats were fed with an 8% NaCl-containing diet (high salt diet) or with a 0.3% NaCl-containing diet (low salt diet; control) from the age of 6 weeks onward, as described.[4] Using osmotic pumps implanted subcutaneously and replaced every two weeks, Dahl rats were treated between the ages of 11 and 17 weeks with the selective β IPKC inhibitor peptide, TAT₄₇₋₅₇- β IV5-3 (n=13, 3mg/kg/day), the selective β IIPKC inhibiting peptide, TAT₄₇₋₅₇- β IIV5-3 (n=11, 3mg/kg/day), an equimolar concentration of TAT₄₇₋₅₇ carrier peptide alone (n=13, 1.6mg/kg/day) or with saline (n=13) (Fig. 1A). Subcutaneous pump implantation was performed in 4% vaporized isoflurane-anesthetized rats. This concentration provided deep anaesthesia, allowing mini-pumps implantation without any clinical sign of pain, such as withdrawal reflex. Pump was inserted in the back of animals after making a sub-scapular incision.

Cardiac functions and blood pressure were similar between groups of animals before any drug treatment was initiated. Survival rate, fractional shortening, lung weight/body weight, LV weight/body weight and blood pressure were evaluated in these groups. Note that 50% of the hypertensive Dahl rats die from hypertensive encephalopathy no later than week 15 of the study [10] whereas cardiac dysfunction-induced death occurs by week 20-22 [11]. None of the PKC inhibitors except for the delta PKC inhibitor inhibited hypertensive encephalopathy and death [10]. Further, the delta PKC inhibitor had no effect on cardiac dysfunction [11]. Further, examination of the brains of the animals that remained alive beyond 15 weeks did not show any pathology, there was only modest renal pathology in the hypertensive rats and there were no differences between the various treatment groups [not shown]. All animal protocols were approved by the Stanford University Institutional Animal Care and Use Committee.

2.3. Cardiovascular measurements

Systolic blood pressure was determined non-invasively using a computerized tail-cuff system (BP-2000, Visitech System, Apex, NC). Non-invasive cardiac function was assessed by two-dimensional guided M-mode echocardiography, in 1% vaporized isoflurane-anesthetized rats after the experimental period. Transthoracic echocardiography was performed using a GE Vivid 7 echocardiograph equipped with a 14 MHz linear transducer.

2.4. Myocardial fibrosis, cardiomyocyte hypertrophy and infiltration of inflammatory cells

Paraffin embedded mid-ventricular sections were deparaffinized, rehydrated and further stained with Masson's trichrome stain. Collagen deposition, an index of myocardial fibrosis, was observed and quantified by measuring the blue stain in the extracellular space (Adobe Photoshop). At least 15 fields (400X) per slide were counted for each group. Photomicrographs of hematoxylin-eosin stained sections (400X) were used to measure the diameter of cardiomyocytes. The myocytes which had the nucleus at their center have been selected for the measurement. At least 150 such myocytes were scored for each group. To evaluate infiltration of inflammatory cells, we again used photomicrographs of hematoxylin-eosin stained sections (400X) and at least 15 fields were counted from each heart.

2.5. Isolated perfused rat heart model

Dahl rats (17-19 weeks old) were injected with heparin (2000U/kg IP) and anesthesia was induced with sodium pentobarbital (100mg/kg IP). The hearts were rapidly excised and then perfused with an oxygenated Krebs-Henseleit solution containing NaCl 120mM, KCl 5.8mM, NaHCO₃ 25mM, NaH₂PO₄ 1.2mM, MgSO₄ 1.2mM, CaCl₂ 1.0mM, and dextrose 10mM, pH 7.4, at 37°C in a Langendorff coronary perfusion system at a constant flow rate of 10mL/min. A thin latex balloon filled with water was inserted into the left ventricle (LV) and connected to a pressure transducer (PowerLab/8SP, AD Instruments) for measurement of the isovolumic LV pressure. The LV volume was adjusted initially at an end-diastolic pressure (EDP) of 5mmHg. All peptides were administered five minutes following a ten minutes equilibration period and the measurements were taken before and after 5 minutes of peptide delivery.

2.6. Isolation of adult rat cardiomyocytes

After the measurement of LV pressure as described above, the control non-treated hearts were further perfused with 2mM EGTA containing low-Ca²⁺ solution 1 (100mM NaCl, 10 mM KCl, 1.2 mM KH₂PO₄, 5 mM MgSO₄, 20 mM glucose, 50 mM taurine, 10 mM HEPES and 100μM CaCl₂), then with low-Ca²⁺ solution 2 (low-Ca²⁺ solution 1 containing pronase E (8mg/100ml; Sigma), proteinase K (1.7mg/100ml; Sigma), bovine albumin (100mg/100ml, fraction V; Sigma) and 200μM CaCl₂). Following digestion, the ventricles were cut into fragments (2-5 mm³) in low-Ca²⁺ solution 1 at room temperature. Tissue fragments were then transferred to a 50ml beaker containing low Ca²⁺ digestion solution 2 supplemented with collagenase (0.5mg/ml; Sigma) and cells were isolated by stirring the tissue for 10 minutes at 37°C. The cell suspension was then filtered through a nylon sieve and centrifuged for 1 min (at 300–400×g). Cell pellets were washed 3 times in low-Ca²⁺ solution 1.

2.7. Calcium transient measurements

Myocytes were loaded with 0.5μM Fura2-acetoxymethyl ester (Molecular Probes, Eugene, Oregon) for 15min, washed and allowed to rest for an additional 40 min to allow the de-esterification of the dye. Myocytes were stimulated at 0.5 Hz and superfused with a HEPES-buffered solution at 25°C. Cells were continuously alternatively excited at 340 and 380nm, at rates as high as 250 pairs/sec using a HyperSwitch system (IonOptix, Milton, MA). Background-corrected Fura2 ratios, reflecting intracellular calcium concentration [12, 13] were collected at 510nm.

2.8. Western blot analysis

At the end of the treatment protocols, the levels and translocation of βI and βIIPKC in LV tissues from 17-week-old Dahl rats were determined by Western blot analysis (n=6/group). The whole tissue lysate, soluble fractions (cytosolic) and particulate fractions (Triton

soluble) were prepared, as previously described [14]. Samples were separated on SDS-polyacrylamide gels by electrophoresis and the proteins were transferred to immobilon-P membranes (Millipore, Billerica, MA). Anti- β IPKC, β IIPKC and G α o rabbit polyclonal antibodies, anti-Collagen-I goat antibodies, anti-GAPDH mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-troponin I and anti-phospho ser23/24 troponin I polyclonal antibodies (Cell Signaling, Danvers, MA), anti-SERCA2 and anti-NCX mouse monoclonal antibodies (Affinity BioReagents, Golden, CO) were used for immunoblotting followed by secondary probing with HRP-conjugated goat anti-rabbit, mouse or goat IgG antibody. All data were normalized to internal controls of GAPDH or G α o.

2.9. Statistics

Data are expressed as the mean $\pm$ SEM. Statistical analysis was assessed by one-way analysis of variance (ANOVA) with *post-hoc* testing by Fisher, two-way repeated measures analysis of variance (ANOVA) with *post-hoc* testing by Fisher or Student's *t*-test (one-tailed distribution/two-sample equal variance) when appropriate. Survival was analyzed by the standard Kaplan-Meier analysis with log-rank test. A value of $P < 0.05$ was considered significant. Note that comparisons conducted at each time point after the start of treatment include only those rats which are still alive at those time points. Therefore, there is inherent survivorship bias in these comparisons.

3. Results

3.1. Inhibition of cardiac hypertrophy, suppression of myocardial fibrosis and inflammation, improvement of cardiac function and prolongation of survival by pharmacological inhibition of β IIPKC in a hypertension-induced heart failure rat model

Since the expression and activity of β PKC increases during heart failure in humans as well as in hypertension-induced cardiac dysfunction in Dahl rats [2-4], we determined the effect of β PKC inhibition on the development of cardiac dysfunction in hypertensive Dahl rats. When placed on a high-salt diet from the age of 6 weeks, the hypertensive Dahl rats (230 vs. 160 mmHg) [4] develop compensatory left ventricular hypertrophy by the age of 11 weeks, and die from cardiac dysfunction between 16 to 21 weeks [15]. Using Alzet pump, we delivered PKC peptide inhibitors in a sustained fashion at 3mg/kg/day. Since there were no differences between TAT and saline control-treated groups [11], analyses were done only with the hypertensive TAT-treated group. Delivery of β IIV5-3 between weeks 11-17 prolonged the survival of hypertensive Dahl rats by about 6 weeks (Fig. 1A and B), diminished end-systolic diameter (Table 1), prevented the decrease in fractional shortening (Fig. 1C) and reduced systolic blood pressure from 254 to 213mmHg at the end of the treatment (17 weeks; Fig. 1D). As expected, β IIV5-3 treatment inhibited β IIPKC activity, but had no effect on β IPKC in these rats (Fig. 1E and F). Conversely, β IV5-3 treatment almost abolished β IPKC activation, but did not significantly affect β IIPKC activation (Fig. 1E and F). *In vivo* sustained treatment with either β IV5-3 or β IIV5-3 did not affect β IPKC and β IIPKC total levels (Fig. 1F) or translocation of other PKC isozymes (Supplement Fig. S1).

β IIV5-3 treatment also significantly decreased LVW/BW ratio groups (Table 1), cardiomyocytes diameter (Fig. 2A), cardiac collagen-I levels (Fig. 2B), area of fibrosis groups (Fig. 2C) and infiltration of inflammatory cells (Fig. 2D) at week 17 relative to hypertensive TAT-treated animals. In contrast, β IV5-3 did not significantly affect any of these parameters (Fig. 2A-D). Therefore, treatment of hypertensive Dahl rats with β IIV5-3 improved systolic function and increased survival probably due to diminished β IIPKC activation (Fig. 1B and C).

3.2. Sustained β IIPKC-inhibition corrects changes in calcium handling proteins and contractile proteins in hypertensive Dahl rats

Previous studies showed that SERCA2 sequesters Ca^{2+} from the cytosol into the sarcomeric reticulum and is down-regulated during heart failure.[16] SERCA2 levels decreased also in the hypertensive rats and treatment with β IIV5-3 *in vivo* restored SERCA2 levels to normal (Fig. 3A). Up-regulation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), which causes sarcoplasmic reticulum calcium depletion in myocytes during heart failure [17-19], was found in the hypertensive hearts and decreased with either β IIV5-3 or β IV5-3 treatments (Fig. 3B). As the sarcoplasmic Ca^{2+} content depends on Ca^{2+} reuptake by SERCA2 relative to transsarcolemmal Ca^{2+} elimination by NCX, we calculated the SERCA2: NCX ratio for all rats studied (Fig. 3C). SERCA2:NCX ratio decreased in the hypertensive rats and treatment with β IIV5-3 *in vivo* restored SERCA2: NCX ratio to normal levels. Cardiac troponin I blocks the contractile function of myofilaments during increased Ca^{2+} transients, which is enhanced through a mechanism involving phosphorylation of Ser23/24 [20, 21]. Troponin phosphorylation in 17 week old hypertensive Dahl rats also increased and selective inhibition of β IIPKC by β IIV5-3 treatment blocked this phosphorylation (Fig. 3D). Therefore, selective inhibition of β IIPKC in hypertensive Dahl rats during the six week period of transition to cardiac failure corrected hypertension-induced changes in the calcium handling protein SERCA2, NCX and troponin I. No changes were observed in either phospholamban levels or its phosphorylated form at serine 16 (Fig. 3E and F).

3.3. Improved contractility of isolated *ex vivo* perfused hearts and a more efficient calcium handling in isolated cardiomyocytes following acute inhibition of β IIPKC

Impaired Ca^{2+} handling has been previously reported in an *ex vivo* Dahl rat heart failure model [16]. Here we found that acute treatment of isolated normotensive Dahl rat hearts in Langendorff mode with β IIV5-3 increased LV end systolic pressure. LV end diastolic pressure decreased, thus resulting in a 30% increase in LV developed pressure relative to the non-treated group (Fig. 4A-F). Importantly, selective inhibition of β IIPKC (by treating with β IV5-3 for the same duration) had no effect on contractility of isolated *ex vivo* perfused hearts (Fig. 4).

Since β IIV5-3 increased cardiac performance in an *ex vivo* model (Fig. 4) and *in vivo* (Fig. 1), we tested the hypothesis that this PKC isozyme may contribute to the impairment of cardiac contractility by alterations in calcium handling. Cytosolic Ca^{2+} transients were measured in cardiomyocytes isolated from normotensive rats and treated with or without the β IIPKC-specific inhibitor, β IIV5-3. *In vitro*, β IIV5-3 treatment resulted in an increase in the total amount of released Ca^{2+} (Ca^{2+} release velocity and peak Ca^{2+} of baseline, respectively; Fig. 5A and B) and the velocity of calcium uptake (Fig. 5C). In contrast, selective inhibition of β IIPKC had no effect on Ca^{2+} transient measurements (Supplement Fig. S2). We did not measure Ca^{2+} transients in failed hearts, since the integrity of the cardiomyocytes after isolation was lost. Nevertheless, improvement of cardiac Ca^{2+} handling in hearts isolated from normotensive rats and treated with the β IIPKC-specific inhibitor, β IIV5-3, supports its contribution to reversing Ca^{2+} handling dysfunction in HF.

4. Discussion

Using an *in vivo* model of cardiac dysfunction (late-phase hypertrophy), we demonstrate that sustained treatment with a β IIPKC-specific peptide inhibitor, β IIV5-3, improved survival and cardiac contractility (Fig. 1 and Table 1), and reduced myocyte hypertrophy, cardiac fibrosis and inflammation (Fig. 2) in a rat model of hypertension-induced cardiac dysfunction. Cardiac hypertrophy is associated with impaired Ca^{2+} handling [16, 22, 23]. As expected, we found a decrease in SERCA levels and an increase in levels of the Na/Ca

exchanger in this model of cardiac dysfunction and β IIPKC inhibition corrected these changes (Fig. 3). Furthermore, we found that acute treatment with the β IIPKC inhibitor *in vitro* increased Ca^{2+} release and uptake (Fig. 5). This effect on calcium handling may be due to β IIPKC-mediated regulation of the L-type Ca^{2+} channel [24] and/or the ryanodine receptor to control Ca^{2+} release and SERCA2 and/or NCX to control Ca^{2+} uptake [17, 18]. There is an alternatively-spliced form of β IIPKC in the hearts that differs only in the last 50 amino acids of the 750 amino acid enzymes. Yet, selective inhibition of β IIPKC had no effect on any of these parameters. These data suggest a selective role for β IIPKC in hypertension-induced cardiac dysfunction.

PKC has been previously shown to regulate the sensitivity of myofilaments to calcium through phosphorylation of troponin I at three different sites (Ser43, Ser45 and Thr144), resulting in diminished contractility of the sarcomeric filament [25, 26]. Thus, β IIPKC and ϵ PKC has been demonstrated to phosphorylate troponin I on Ser23/24, which are well-known PKA-sensitive sites [20, 21, 25]. Using the β IIPKC and β IIPKC-selective inhibitors, we showed that treatment of the hypertensive Dahl rats with β IIIV5-3 (and not with the β IIPKC inhibitor) significantly decreased troponin I phosphorylation on Ser23/24 (Fig. 3C), suggesting that the improved cardiac function and contractility associated with β IIIV5-3 treatment may be due to both the partial correction of the heart failure-induced impaired Ca^{2+} handling and to an increase in Ca^{2+} sensitivity of the cardiac myofilaments. However, the direct and/or indirect contribution of β IIPKC to troponin I phosphorylation at the different PKC-sensitive sites as well as the precise mechanism of PKC-mediated troponin I phosphorylation remain to be fully established. A role for β IIPKC (and perhaps also β IIPKC) in regulating the levels of SERCA2 and NCX has also been shown in this hypertension-induced cardiac dysfunction model (Fig. 3A and B).

Cardiac fibrosis is considered the major determinant of diastolic dysfunction and failure in hypertension [27], since it leads to passive stiffness, impairment of relaxation and diastolic filling. Continuous accumulation of collagen interferes in cardiomyocytes contractility whereas alteration in collagen metabolism significantly contributes to ventricular dysfunction [27-29]. Similar to human failing heart findings [30], in the present study we found a pronounced increase in fibrosis and collagen type-1 level in the myocardium from hypertensive rats. Further, treatment with β IIIV5-3 attenuated perivascular collagen deposition (Fig. 2C) and collagen-1 levels in the myocardium. In general, attenuation of perivascular fibrosis improves oxygen supply and removal of waste metabolites and ultimately the cardiac function. *In vitro* studies indicated that different PKC isozymes participate in different stages of fibrotic [31]. This *in vivo* study reaffirms that suppression of cardiac fibrosis by specifically inhibiting β IIPKC from hypertrophic stage is an important mechanism to prevent myocardial dysfunction and failure in hypertensive heart failure. The cellular mechanisms underlying the decrease in cardiac fibrosis mediated by β IIPKC inhibition are out of the scope of the present study. Another important contributing factor for heart failure pathogenesis is inflammation. We and others have shown that PKC isozymes are involved in this phenomenon [31]. However, further studies on the role of PKC isozymes in the activation of specific inflammatory cells during cardiac remodeling are needed.

Further, it is clear that β IIPKC phosphorylates multiple protein substrates, each of which may have different role in heart failure development, perhaps even at different time-point of the disease. However, the available pharmacological tools cannot individually inhibit the regulation of each of these substrates. Inhibitors that will prevent β IIPKC-mediated phosphorylation of each protein substrates, so called separation-of-function inhibitors, will identify which of the many substrates of β IIPKC is critical in a given response and at what time during the development of heart failure.

Importantly, although the heart expresses several PKC isozymes, β IIPKC seems to be the key effector isozyme in the failing human hearts [2]. These human data are in agreement with the data we described here in rats. However, it should be noted that there is a controversy about the role of β PKC in the mouse heart. Roman *et al.* (2001) [32] showed no cardiac phenotype in β PKC KO mice, whereas overexpression of β IIPKC induced cardiac hypertrophy [33] and contractility dysfunction [34]. These studies, examining the role of β PKC, were done in mice and therefore may reflect species differences. Further, benefits of β PKC inhibition in diabetes-induced cardiomyopathy in rats were reported by several groups [35, 36], suggesting perhaps difference between mice and rats regarding the role of PKC isozymes in cardiac pathology.

In the current study, we selected β I- and β IIPKC, because in our previous studies we found that their levels were elevated in the end stage failed hearts from hypertensive Dahl rats [4]. In addition to the current study, α -, β II- and ϵ PKCs have been evaluated as targets for heart failure drugs by chronic pharmacological methods [11, 37, 38]. Using the same model, we previously reported that ϵ PKC activation occurs early during the compensatory stage (11-17 weeks) and sustained inhibition of ϵ PKC attenuated the development of cardiac fibrosis and dysfunction in hypertension-induced heart failure [11]. α PKC has also been suggested as a therapeutic candidate for the treatment of heart failure based on studies in mice [11, 37, 39]. However, the inhibitors used in those studies may inhibit several or all of the four classical PKC isozymes [39, 40].

5. Conclusion

In conclusion, an isozyme-specific inhibitor for β IIPKC, β IIV5-3, improved cardiac contractility *ex vivo* in normal and failing hearts and *in vivo* in failing hearts. Further, whereas β IPKC inhibition *in vivo* was without significant effects, the selective inhibitor of β IIPKC attenuated cardiac hypertrophy, myocardial fibrosis, cardiac inflammation, slowed down the progression of cardiac dysfunction and prolonged survival of rats. Together, we found that the β IIPKC-mediated pathological effects may result, in part, by inducing calcium handling defect, increasing fibrosis and increasing inflammation in the failing heart. Considering that PKC β II phosphorylates multiple protein substrates, other cellular and molecular events may also contribute to β IIPKC-mediated role in failed hearts at different time of the disease development. Further, PKC β II over-activation may contribute indirectly to some the pathologies associated with heart failure. Finally, an important question not addressed in the present study is related to the time course of PKC β II activation following the onset of hypertension, which can help identify the optimal time window for drug therapy with a selective β IIPKC inhibitor. Further, whether our findings in these hypertensive rats are translatable to other models of heart failure have yet to be determined. Because similar to this rat model of cardiac dysfunction, failed human hearts also have a selective increase in β PKC [2, 3], our data suggest that a selective inhibitor of β IIPKC function may have a therapeutic potential in the treatment of human heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Thom T, Haase N, Rosamond W, Howard VJ, Rumsfeld J, Manolio T, et al. Heart disease and stroke statistics--2006 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation*. 2006; 113:e85–151. [PubMed: 16407573]
2. Bowling N, Walsh RA, Song G, Estridge T, Sandusky GE, Fouts RL, et al. Increased protein kinase C activity and expression of Ca²⁺-sensitive isoforms in the failing human heart. *Circulation*. 1999; 99:384–91. [PubMed: 9918525]
3. Simonis G, Briem SK, Schoen SP, Bock M, Marquetant R, Strasser RH. Protein kinase C in the human heart: differential regulation of the isoforms in aortic stenosis or dilated cardiomyopathy. *Mol Cell Biochem*. 2007; 305:103–11. [PubMed: 17594058]
4. Inagaki K, Iwanaga Y, Sarai N, Onozawa Y, Takenaka H, Mochly-Rosen D, et al. Tissue angiotensin II during progression or ventricular hypertrophy to heart failure in hypertensive rats; differential effects on PKC epsilon and PKC beta. *J Mol Cell Cardiol*. 2002; 34:1377–85. [PubMed: 12392998]
5. Stebbins EG, Mochly-Rosen D. Binding specificity for RACK1 resides in the V5 region of beta II protein kinase C. *Journal of Biological Chemistry*. 2001; 276:29644–50. [PubMed: 11387319]
6. Souroujon MC, Mochly-Rosen D. Peptide modulators of protein-protein interactions in intracellular signaling. *Nat Biotechnol*. 1998; 16:919–24. [PubMed: 9788346]
7. Churchill E, Budas G, Vallentin A, Koyanagi T, Mochly-Rosen D. PKC Isozymes in Chronic Cardiac Disease: Possible Therapeutic Targets? *Annu Rev Pharmacol Toxicol*. 2007
8. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF. In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science*. 1999; 285:1569–72. [PubMed: 10477521]
9. Chen L, Wright LR, Chen CH, Oliver SF, Wender PA, Mochly-Rosen D. Molecular transporters for peptides: delivery of a cardioprotective epsilonPKC agonist peptide into cells and intact ischemic heart using a transport system, R(7). *Chem Biol*. 2001; 8:1123–9. [PubMed: 11755391]
10. Qi X, Inagaki K, Sobel RA, Mochly-Rosen D. Sustained pharmacological inhibition of deltaPKC protects against hypertensive encephalopathy through prevention of blood-brain barrier breakdown in rats. *J Clin Invest*. 2008; 118:173–82. [PubMed: 18097471]
11. Palaniyandi SS, Inagaki K, Mochly-Rosen D. Mast cells and epsilonPKC: a role in cardiac remodeling in hypertension-induced heart failure. *J Mol Cell Cardiol*. 2008; 45:779–86. [PubMed: 18804478]
12. Nagata K, Liao R, Eberli FR, Satoh N, Chevalier B, Apstein CS, et al. Early changes in excitation-contraction coupling: transition from compensated hypertrophy to failure in Dahl salt-sensitive rat myocytes. *Cardiovasc Res*. 1998; 37:467–77. [PubMed: 9614501]
13. Silver RB. Ratio imaging: measuring intracellular Ca⁺⁺ and pH in living cells. *Methods Cell Biol*. 2003; 72:369–87. [PubMed: 14719341]
14. Churchill EN, Ferreira JC, Brum PC, Szweda LI, Mochly-Rosen D. Ischaemic preconditioning improves proteasomal activity and increases the degradation of deltaPKC during reperfusion. *Cardiovasc Res*. 2010; 85:385–94. [PubMed: 19820255]
15. Iwanaga Y, Kihara Y, Hasegawa K, Inagaki K, Yoneda T, Kaburagi S, et al. Cardiac endothelin-1 plays a critical role in the functional deterioration of left ventricles during the transition from compensatory hypertrophy to congestive heart failure in salt-sensitive hypertensive rats. *Circulation*. 1998; 98:2065–73. [PubMed: 9808606]
16. Seki S, Nagai M, Takeda H, Onodera T, Okazaki F, Taniguchi M, et al. Impaired Ca²⁺ handling in perfused hypertrophic hearts from Dahl salt-sensitive rats. *Hypertension Research - Clinical & Experimental*. 2003; 26:643–53.
17. Houser SR, Piacentino V 3rd, Weissner J. Abnormalities of calcium cycling in the hypertrophied and failing heart. *Journal of Molecular & Cellular Cardiology*. 2000; 32:1595–607. [PubMed: 10966823]
18. Hoshijima M. Gene therapy targeted at calcium handling as an approach to the treatment of heart failure. *Pharmacology & Therapeutics*. 2005; 105:211–28. [PubMed: 15737405]
19. Bartholomeu JB, Vanzelli AS, Rolim NP, Ferreira JC, Bechara LR, Tanaka LY, et al. Intracellular mechanisms of specific beta-adrenoceptor antagonists involved in improved cardiac function and

- survival in a genetic model of heart failure. *J Mol Cell Cardiol.* 2008; 45:240–9. [PubMed: 18632114]
20. Kobayashi T, Yang X, Walker LA, Van Breemen RB, Solaro RJ. A non-equilibrium isoelectric focusing method to determine states of phosphorylation of cardiac troponin I: identification of Ser-23 and Ser-24 as significant sites of phosphorylation by protein kinase C. *J Mol Cell Cardiol.* 2005; 38:213–8. [PubMed: 15623438]
 21. Itoh S, Ding B, Bains CP, Wang N, Takeishi Y, Jalili T, et al. Role of p90 ribosomal S6 kinase (p90RSK) in reactive oxygen species and protein kinase C beta (PKC-beta)-mediated cardiac troponin I phosphorylation. *J Biol Chem.* 2005; 280:24135–42. [PubMed: 15840586]
 22. Ferreira JC, Moreira JB, Campos JC, Pereira MG, Mattos KC, Coelho MA, et al. Angiotensin receptor blockade improves the net balance of cardiac Ca(2+) handling-related proteins in sympathetic hyperactivity-induced heart failure. *Life Sci.* 2011; 88:578–85. [PubMed: 21277865]
 23. Pereira MG, Ferreira JC, Bueno CR Jr. Mattos KC, Rosa KT, Irigoyen MC, et al. Exercise training reduces cardiac angiotensin II levels and prevents cardiac dysfunction in a genetic model of sympathetic hyperactivity-induced heart failure in mice. *Eur J Appl Physiol.* 2009; 105:843–50. [PubMed: 19125280]
 24. Zhang ZH, Johnson JA, Chen L, El-Sherif N, Mochly-Rosen D, Boutjdir M. C2 region-derived peptides of beta-protein kinase C regulate cardiac Ca2+ channels. *Circ Res.* 1997; 80:720–9. [PubMed: 9130453]
 25. Layland J, Solaro RJ, Shah AM. Regulation of cardiac contractile function by troponin I phosphorylation. *Cardiovasc Res.* 2005; 66:12–21. [PubMed: 15769444]
 26. Wang H, Grant JE, Doede CM, Sadayappan S, Robbins J, Walker JW. PKC-betaII sensitizes cardiac myofilaments to Ca2+ by phosphorylating troponin I on threonine-144. *J Mol Cell Cardiol.* 2006; 41:823–33. [PubMed: 17010989]
 27. Burlew BS, Weber KT. Cardiac fibrosis as a cause of diastolic dysfunction. *Herz.* 2002; 27:92–8. [PubMed: 12025467]
 28. Diez J, Lopez B, Gonzalez A, Querejeta R. Clinical aspects of hypertensive myocardial fibrosis. *Curr Opin Cardiol.* 2001; 16:328–35. [PubMed: 11704701]
 29. Plaksej R, Kosmala W, Frantz S, Herrmann S, Niemann M, Stork S, et al. Relation of circulating markers of fibrosis and progression of left and right ventricular dysfunction in hypertensive patients with heart failure. *J Hypertens.* 2009; 27:2483–91. [PubMed: 19887955]
 30. Querejeta R, Lopez B, Gonzalez A, Sanchez E, Larman M, Martinez Ubago JL, et al. Increased collagen type I synthesis in patients with heart failure of hypertensive origin: relation to myocardial fibrosis. *Circulation.* 2004; 110:1263–8. [PubMed: 15313958]
 31. Palaniyandi SS, Sun L, Ferreira JC, Mochly-Rosen D. Protein kinase C in heart failure: a therapeutic target? *Cardiovasc Res.* 2009; 82:229–39. [PubMed: 19168855]
 32. Roman BB, Geenen DL, Leitges M, Buttrick PM. PKC-beta is not necessary for cardiac hypertrophy. *American journal of physiology.* 2001; 280:H2264–70. [PubMed: 11299230]
 33. Wakasaki H, Koya D, Schoen FJ, Jirousek MR, Ways DK, Hoit BD, et al. Targeted overexpression of protein kinase C beta2 isoform in myocardium causes cardiomyopathy. *Proc Natl Acad Sci U S A.* 1997; 94:9320–5. [PubMed: 9256480]
 34. Bowman JC, Steinberg SF, Jiang T, Geenen DL, Fishman GI, Buttrick PM. Expression of protein kinase C beta in the heart causes hypertrophy in adult mice and sudden death in neonates. *J Clin Invest.* 1997; 100:2189–95. [PubMed: 9410895]
 35. Arikawa E, Ma RC, Isshiki K, Luptak I, He Z, Yasuda Y, et al. Effects of insulin replacements, inhibitors of angiotensin, and PKCbeta's actions to normalize cardiac gene expression and fuel metabolism in diabetic rats. *Diabetes.* 2007; 56:1410–20. [PubMed: 17363743]
 36. Connelly KA, Kelly DJ, Zhang Y, Prior DL, Advani A, Cox AJ, et al. Inhibition of protein kinase C-beta by ruboxistaurin preserves cardiac function and reduces extracellular matrix production in diabetic cardiomyopathy. *Circ Heart Fail.* 2009; 2:129–37. [PubMed: 19808328]
 37. Hambleton M, Hahn H, Pleger ST, Kuhn MC, Klevitsky R, Carr AN, et al. Pharmacological- and gene therapy-based inhibition of protein kinase C α /beta enhances cardiac contractility and attenuates heart failure. *Circulation.* 2006; 114:574–82. [PubMed: 16880328]

38. Palaniyandi SS, Ferreira JC, Brum PC, Mochly-Rosen D. PKC β II inhibition attenuates myocardial infarction induced heart failure and is associated with a reduction of fibrosis and pro-inflammatory responses. *J Cell Mol Med.* 2011; 15:1769–77. [PubMed: 20874717]
39. Hahn HS, Marreez Y, Odley A, Sterbling A, Yussman MG, Hilty KC, et al. Protein kinase C α negatively regulates systolic and diastolic function in pathological hypertrophy. *Circ Res.* 2003; 93:1111–9. [PubMed: 14605019]
40. Wilkinson SE, Parker PJ, Nixon JS. Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem J.* 1993; 294(Pt 2):335–7. [PubMed: 8373348]
41. Iwanaga Y, Kihara Y, Inagaki K, Onozawa Y, Yoneda T, Kataoka K, et al. Differential effects of angiotensin II versus endothelin-1 inhibitions in hypertrophic left ventricular myocardium during transition to heart failure. *Circulation.* 2001; 104:606–12. [PubMed: 11479261]

Highlights

- Selective β IIPKC inhibition improves cardiac function and survival in heart failure.
- β IIPKC inhibition reverses pathological cardiac remodeling.
- β IIV5-3 peptide suppresses myocardial fibrosis and inflammation.
- β IIPKC inhibition improves calcium handling and myocardial contractility.

Figure 1. β IIV5-3, but not β IV5-3, slowed down the progression of cardiac dysfunction

(A): Schematic panel of pharmacological treatment in Dahl rats. (B): Survival rate of hypertensive Dahl rats after six weeks with either TAT carrier peptide (TAT, n=13) or the β PKC inhibitors, β IV5-3 (n=13) or β IIV5-3 (n=11). The shaded area represents the duration of treatment. (C): Fractional shortening. (D): Systolic blood pressure measured at the age of 11, 13, 15 and 17 weeks (n=6-17 per group). (E): Presented are total β IPKC and β IIPKC levels and their relative levels in the particulate fraction (expressed as the ratio of levels in Triton-soluble over total fraction) in 17-week old rats (n=6 per group). The level of cellular PKC distribution between the total and particulate fractions was normalized using GAPDH and $G\alpha_o$, respectively. Note that β IPKC translocation changed significantly in hypertensive hearts. However, its sustained inhibition had no effect on survival and cardiac function. (F): Representative blot showing the levels and subcellular distribution of β IIPKC in 17-week old rats (n=6 per group). * P <0.05 vs. normotensive control (NT-C). § P <0.05 vs. hypertensive TAT-treated animals. † P <0.05 vs. hypertensive TAT and β IV5-3-treated animal. Survival was analyzed by the standard Kaplan-Meier analysis. Fractional shortening, total β IPKC and β IIPKC levels and translocations were analyzed by the one-way analysis of variance (ANOVA) with *post-hoc* testing by Fisher. NT-CTL, normotensive rats; HT-TAT, hypertensive rats; HT- β IV5-3 and HT- β IIV5-3, hypertensive rats treated with specific β IPKC and β IIPKC inhibitor, respectively.

Figure 2. Treatment with β IIV5-3, but not with β IV5-3, reduced cardiomyocyte hypertrophy, fibrosis and infiltration of inflammatory cells

(A): Presented are data on averaged cardiomyocyte diameters. Myocytes whose nuclei were at the center of the cells were selected for the measurement. At least 150 such myocytes from each group were measured. $^{\dagger}P < 0.05$ vs. HT-TAT. (B): Quantification of collagen-I levels in hearts treated with either β PKC inhibitors were compared with hearts control peptide (100%). Protein levels are normalized to an internal loading control (GAPDH). Representative blots are presented in the lower panels. $^{\dagger}P < 0.05$ vs. HT-TAT; $^{\ddagger}P < 0.05$ vs. HT- β IV5-3. (C): Representative photomicrographs (bar=200 μ m) and average of collagen deposition by Masson's trichrome stain. (D): Infiltration of inflammatory cells at the perivascular region was assessed using Hematoxylin-eosin stain (400X). The number of infiltrating cells was quantified. $^{\dagger}P < 0.05$ vs. HT-TAT. One-way analysis of variance (ANOVA) with *post-hoc* testing by Fisher was used in those analyses. NT, normotensive rats; HT-TAT, hypertensive rats; HT- β IV5-3 and HT- β IIV5-3, hypertensive rats treated with specific β IPKC and β IIPKC inhibitor, respectively.

Figure 3. Phosphorylation and levels of proteins involved in cardiac contractility are regulated by βIIV5-3 treatment

(A): SERCA2, (B): $\text{Na}^+/\text{Ca}^{2+}$ exchanger, (C) SERCA2: $\text{Na}^+/\text{Ca}^{2+}$ exchanger ratio, (D): phosphorylated troponin I, (E) phospholamban and (F) phosphorylated phospholamban levels in normotensive (NT) and hypertensive (HT) rats are presented after normalization with GAPDH or total troponin I level. (G) Representative blots. * $P < 0.05$ vs. HT-TAT, § $P < 0.05$ vs. NT-CTR, HT-βIV5-3 and HT-βIIV5-3. One-way analysis of variance (ANOVA) with *post-hoc* testing by Fisher was used in those analyses. NT-CTL, normotensive rats; HT-TAT, hypertensive rats; HT-βIV5-3 and HT-βIIV5-3, hypertensive rats treated with specific βIPKC and βIIPKC inhibitor, respectively.

Figure 4. Treatment with β IIV5-3, but not with β IV5-3, caused better contractility in isolated *ex vivo* perfused heart

Isolated hearts from normotensive Dahl rats were perfused on a Langendorff apparatus with Krebs's buffer. $1\mu\text{M}$ of either β IV5-3 or β IIV5-3 was perfused in the buffer for ten minutes, Left ventricular (LV) end systolic pressure (LVESP, A) and LV end diastolic pressure (LVEDP, B) were measured. LV developed pressure (LVDP, C) was measured by subtracting LV end diastolic pressure from LV end systolic pressure. Comparison of LVDP from the non-treated independent control and β IIV5-3 treated hearts is shown in D. Squares, triangles and circles represent the values for individual hearts. $+dP/dT$ (E) and $-dP/dT$ (F) values were recorded from the same experiment as described above. * $P < 0.05$ vs. before

treatment. ‡ $P < 0.05$ vs. β IV5-3 treatment. One-way analysis of variance (ANOVA) with *post-hoc* testing by Fisher was used to compare the effect of β IPKC or β IIPKC on LVESP, LVEDP, LVDP, positive and negative left ventricular dP/dT.

Figure 5. Treatment with βIIV5-3, resulted in better Ca²⁺ handling in isolated myocytes
 Intracellular Ca²⁺ transient in isolated ventricular myocytes. Ventricular cardiomyocytes were isolated from normotensive Dahl rats (17-19 weeks old). 1μM of βIIV5-3 was perfused in the buffer for ten minutes. Ca²⁺ trace (340/380nm ratio; A), Ca²⁺ release velocity (ratio/second; B), peak Ca²⁺ (% of baseline; C) and Ca²⁺ uptake velocity (ratio/second; D) **P*<0.05 vs. control. One-way analysis of variance (ANOVA) with *post-hoc* testing by Fisher was used to compare the effect of βIIPKC on Ca²⁺ release velocity, peak Ca²⁺ and Ca²⁺ uptake velocity.

Table 1

Body weight and *in vivo* echocardiographic data in Dahl rats.

Group	n	BW (g)	LVW/BW (mg/g)	PWT (mm)	EDD (mm)	ESD (mm)	SWS (g/cm ²)
NT-CTL	28	424±5	2.3±0.1 [†]	1.6±0.0	7.0±0.1	2.8±0.1 [†]	29.1±3.3 [†]
HT-TAT	9	358±18 [*]	4.5±0.2 [*]	1.6±0.0	7.6±0.2 [*]	4.2±0.2 [*]	82.7±18.5 [*]
HT-βIV5-3	4	373±17 [*]	3.9±0.1 [*]	1.6±0.0	7.4±0.5 [*]	4.0±0.9 [*]	86.8±14.4 [*]
HT-βIIV5-3	9	391±13 [*]	3.5±0.1 ^{*†}	1.6±0.1	7.6±0.1 [*]	3.3±0.3 [†]	46.1±8.1 [†]

Body weight (BW), left ventricular weight/body weight ratio (LVW/BW) and echocardiographic data were measured at 17 weeks of age. PWT = LV posterior wall thickness; EDD = LV end-diastolic diameter; ESD = LV end-systolic diameter. SWS = LV systolic wall stress (SBP × ESD) / [4 × PWT × (1+PWT/ESD)]. Values are mean ± SEM.

NT-CTL, normotensive rats; HT-TAT, hypertensive rats; HT-βIV5-3 and HT-βIIV5-3, hypertensive rats treated with specific βIPKC and βIIPKC inhibitor, respectively. Note that, as previously reported, [41] PWT, which increased by ~30% at 11 weeks, returns to the dimension of normotensive rats by 17 weeks. One-way analysis of variance (ANOVA) with *post-hoc* testing by Fisher was used in those analyses.

^{*} $P < 0.05$ vs. normotensive control (NT-CTL)

[†] $P < 0.05$ vs. hypertensive TAT and βIV5-3-treated animals. (SEM of 0.0 is smaller than 0.05).