Phosphodiesterase 9A controls nitric-oxide-independent cGMP and hypertrophic heart disease

Dong I. Lee1, Guangshuo Zhu1, Takashi Sasaki2, Gun-Sik Cho3, Nazha Hamdani3, Ronald Holewinski4,5, Su-Hyun Je5, Thomas Danner1, Manling Zhang1, Peter P. Rainer1, Djahida Bedja1, Jonathan A. Kirk1, Mark J. Ranek1, Wolfgang R. Dostmann6, Chulan Kwon1, Kenneth B. Margulies7, Jennifer E. Van Eyk1,8, Walter J. Paulus1, Eiki Takimoto1 & David A. Kass1

Cyclic guanosine monophosphate (cGMP) is a second messenger molecule that transduces nitric-oxide- and natriuretic-peptide-coupled signalling, stimulating phosphorylation changes by protein kinase G. Enhancing cGMP synthesis or blocking its degradation by phosphodiesterase type 5A (PDE5A) protects against cardiovascular disease4,5. However, cGMP stimulation alone is limited by counteradaptations including PDE upregulation1. Furthermore, although PDE5A regulates nitric-oxide-generated cGMP4,5, nitric oxide signalling is often depressed by heart disease6. PDEs controlling cGMP remain uncertain. Here we show that cGMP-selective PDE9A (refs 7, 8) is expressed in the mammalian heart, including humans, and is upregulated by hypertrophy and cardiac failure. PDE9A regulates natriuretic-peptide- rather than nitric-oxide-stimulated cGMP in heart myocytes and muscle, and its genetic or selective pharmacological inhibition protects against pathological responses to neurohormones, and sustained pressure-overload stress. PDE9A inhibition reverses pre-established heart disease independent of nitric oxide synthase (NOS) activity, whereas PDE5A inhibition requires active NOS. Transcription factor activation and phosphoproteome analyses of myocytes with each PDE selectively inhibited reveals substantial differential targeting, with phosphorylation changes from PDE5A inhibition being more sensitive to NOS activation. Thus, unlike PDE5A, PDE9A can regulate cGMP signalling independent of the nitric oxide pathway, and its role in stress-induced heart disease potential as a therapeutic target.

The PDE super-family contains 11 sub-genes conferring different cyclic nucleotide and tissue selectivity7. PDE5A was the first cGMP-selective enzyme discovered, and has a major role in erectile and pulmonary vasomotor control. PDE9A was cloned 20 years later7,8 and has the highest affinity and selectivity for cGMP with a Michaelis constant (Km) for cGMP 100-fold lower than for cAMP7. PDE5A and PDE9A share only 28% homology7 and PDE9A lacks amino-terminus cGMP/protein kinase G (PKG) stimulatory regulatory domains present in PDE5A7. PDE9A is expressed primarily in the brain, gut and kidney. To date, studies have focused on its role in cognitive function7,11 and while mRNA is detectable in the heart and other tissues7,11, its role outside of the brain remains largely unknown.

To test PDE9A involvement in the heart, gene and protein expression were assessed in myocardial tissue and isolated myocytes. Figure 1a shows PDE9A immunostaining in rat neonatal cardiomycytes (RNCMs) and adult mouse myocytes, with targeted gene deletion (short interfering RNA (siRNA) or Pde9a−/− mice). Extended Data Fig. 1) as a negative control. Protein detection by immunoblot in neonatal myocytes is shown in Extended Data Fig. 2a. Basal gene expression is low but increases with agonist (for example, phenylephrine) or mechanical (in vivo pressure

**Figure 1 | PDE9A expression in heart and myocytes increases with disease.** a, PDE9A in neonatal and adult cardiomyocytes; gene silencing as negative controls (scale bar, 20 μm). b, Pde9a gene expression in RNCMs with phenylephrine (PE) or adult myocytes/heart (mouse) after TAC. *P < 0.01 vs. control, Ctrl; control, KO, knockout. c, Immunoblot/immunostaining of PDE9A from human dilated cardiomyopathy (DCM) and non-failing (NF) myocardium (scale bar, 200 μm). d, e, PDE9A expression (d) and activity (e) in NF and DCM. f, PDE9A protein expression in human myocardium for NF, HFPEF and aortic stenosis (AS). *P < 0.0001, **P < 0.005 vs. non-failing; ***P < 0.0001 vs. AS and DCM. g−k, Co-localization of cardiac troponin T (cTnT) and PDE9A in human DCM myocardium. j−k, In situ hybridization of PDE9A in human NF and DCM myocardium. Data are mean ± s.e.m. and biological replicates are indicated.

---

1Department of Cardiology, Department of Medicine, The Johns Hopkins Medical Institutions, Baltimore, Maryland 21205, USA. 2Advanced Medical Research Laboratories, Research Division, Mitsubishi Tanabe Pharma Corporation, Yokohama, Kanagawa 227-0033, Japan. 3Department of Physiology, Institute for Cardiovascular Research, VU University Medical Center, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands. 4Heart Institute and Advanced Clinical Biosystems Research Institute, Cedar Sinaia Medical Center, 8700 Beverly Blvd, AHSF A9229 Los Angeles, California 90048, USA. 5Department of Pharmacology, University of Virginia, Charlottesville, Virginia 22903, USA. 6Department of Medicine, Division of Cardiovascular Medicine, Cardiovascular Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA.

©2015 Macmillan Publishers Limited. All rights reserved
overload) stimulation (Fig. 1b). Increased PDE9A protein expression and cGMP-esterase activity is found in left ventricular myocardium from humans with heart failure and depressed function (Fig. 1c–e, Extended Data Table 1). Protein expression also increases in human left ventricular hypertrophy from aortic stenosis (pressure overload) and most notably in heart failure with preserved ejection fraction (HFPEF, Fig. 1f), a prevalent form of heart failure wherein contractile function appears normal despite symptoms\(^1\). Human PDE9A expression primarily localizes to myocytes based on co-localization with troponin-T (Fig. 1g–i) and in situ hybridization staining (Fig. 1j, k). Whereas PDE5A is expressed in fibroblasts\(^9\), PDE9A is essentially undetectable in isolated human fibroblasts (qPCR threshold cycle \(= 39\)).

PDE9A upregulation by heart disease suggested its inhibition might blunt pathological stress responses. To test this, RNCMs and adult myocytes were stimulated with phenylephrine or endothelin-1 (ET-1), increasing protein synthesis and hypertrophic fetal gene (Nppa, Nppb) expression. Co-incubation with a selective PDE9A antagonist (PF-04449613 (PF-9613), 5 \(\mu\)M, Extended Data Fig. 3a for selectivity of PDE9A versus PDE5A), or Pde9a gene silencing/deletion (Fig. 2a, b (top)) reversed these changes. Cells lacking PDE9A were unaffected by PF-9613, confirming the drug’s selectivity (Fig. 2a). Similar results were obtained with PF-04447943, another PDE9A inhibitor now used in human trials (NCT00930059, Extended Data Fig. 3b). Anti-hypertrophic effects of PDE9A inhibition required activation of PKG, as they were blocked by PKG inhibitor DT3 (Fig. 2b (bottom) and Extended Data Fig. 3c).

Both PDE5A and PDE9A regulate cGMP–PKG activity; therefore we tested if this regulation is redundant or targets different cGMP pools. Gene silencing of Pde9a in RNCMs had no effect on cGMP augmentation from a nitric oxide donor (diethylenetriamine (DEANO), 1 \(\mu\)M) but enhanced cGMP (Fig. 2c) and PKG activity (Extended Data Fig. 4a) suggested by natriuretic peptide type A (ANP (also known as NPPA), 1 \(\mu\)M). Adult myocytes exposed to PF-9613 also augmented cGMP only with ANP stimulation (Fig. 2d; Pde9a–/– cells were negative controls). We also measured intracellular cGMP generation in adult myocytes expressing the cGMP–fluorescent sensor, FlincG13 (Fig. 2e).

Figure 2 | PDE9A inhibition suppresses cardiac hypertrophy via natriuretic-peptide–cGMP pathway. a, Effect of PF-9613 or gene silencing on hypertrophic gene activation induced by phenylephrine (PE) or endothelin-1 (ET-1) in RNCMs (\(n = 12\) per group for all but Nppa Pde9a siRNA \(n = 8\) and adult myocytes \(n = 6\)). \#P < 0.05 vs. PE, \*P < 0.01 vs. baseline, \(\dagger\)P < 0.001 vs. baseline.

b, Effect of PF-9613 or gene silencing on PE- or ET-1-stimulated protein synthesis. \#P < 0.05 vs. phenylephrine/ET-1, \*P < 0.01 vs. baseline. Bottom, PKG–inhibitor DT3 prevents anti-hypertrophic effect of PF-9613. \#P < 0.05 vs. baseline. c, DEANO-simulated cGMP from ANP but not DEANO simulation in neonatal and adult myocytes. All groups \#P < 0.01 vs. baseline; \*P < 0.01 vs. ANP. e, Left, FlincG–cGMP fluorescence in RNCM before and after ANP stimulation (colour coded for sampled intracellular location). Right, time course normalized to baseline. f, Myocyte cGMP stimulated by ANP rises with PF-9613 in RNCMs but not cells with Pde9a silenced. g, PF-9613 does not alter DEANO-stimulated cGMP whereas a PDE5A inhibitor (SIL) does. h, ANP \(\pm\) PF-9613 effects are unchanged by ODQ, whereas DEANO \(\pm\) SIL are inhibited. i, RNCMs exposed to L-NNAME and phenylephrine \(\pm\) Pde9a siRNA, Pde9a siRNA, or both. \#P < 0.05 vs. baseline, \*P < 0.05 vs. phenylephrine with scrambled siRNA.

Data are mean ± s.e.m. and biological replicates are indicated.
ANP-stimulated cGMP increased more after PF-9613 in control cells, but not in those lacking PDE9A (Fig. 2f). By contrast, PF-9613 did not alter cGMP stimulated by DEANO, whereas the latter increased with PDE5A-inhibition (sildenafil (SIL), 1 μM, Fig. 2g). Neither SIL nor PDE5A-siRNA altered ANP-stimulated cGMP (Extended Data Fig. 4b, c). Pre-incubation with soluble guanylate cyclase (sGC) inhibitor ODQ (10 μM, 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one) had no effect on cGMP stimulated by ANP or ANP plus PF9613, but fully blocked the rise from DEANO or DEANO on cGMP stimulated by ANP or ANP plus PF9613, but fully blocked (10 μM, 1H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-1-one) had no effect on cGMP stimulated by ANP or ANP plus PF9613, but fully blocked.

We hypothesized that disparate cGMP targeting is related to intracellular localization and thus PDE compartmentation. Confocal immunochemistry confirmed this, revealing PDE5A but not PDE9A co-localization with α-actinin at the Z-disc, and PDE9A but not PDE5A co-localization with T-tubular membranes (sarcoplasmic reticulum ATPase-2a) (Extended Data Fig. 5). Interestingly, natriuretic peptide receptor type A also displays a striation pattern in myocytes and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation. Confocal immunohistochemistry confirmed this, revealing PDE5A but not PDE9A cellular localization and thus PDE compartmentation.

The clinically relevant question is whether inhibition of PDE9A reverses pre-established hypertrophy/dysfunction, and whether this is differentiable from the protection afforded by PDE5A inhibition. To address this, C57BL/6J mice were subjected to severe TAC to rapidly chamber dilation, dysfunction and hypertrophy. On day 8, mice were divided into three groups: PF-9613, SIL or vehicle, and treated for an additional 4 weeks while maintaining TAC. Oral PF-9613 itself had no effect on blood pressure or heart function (Extended Data Fig. 7). To test if cGMP targeting by PDE9A differed from PDE5A in vivo, the experiment was repeated adding the NOS inhibitor L-NAME to the drinking water.

All groups developed marked chamber dysfunction, dilation, and hypertrophy after 1 week of TAC (pre-treatment; Fig. 4a, b). In mice that did not receive L-NAME, inhibiting either PDE reversed changes to near sham-control levels and lowered post-mortem left ventricular mass, lung weight and abnormal molecular signatures (Extended Data Fig. 8). However, in L-NAME-treated mice, only PDE9A inhibition was effective (Fig. 4a, b). Blocking either PDE increased myocardial cGMP in L-NAME + TAC but only PDE9A inhibition did so in L-NAME − TAC mice (Fig. 4c). L-NAME prevented PKG activation by SIL but not PF-9613 (Fig. 4d). In vitro PKG activity in PDE9A-treated myocardium was itself little altered. This parallels reported data with natriuretic peptide stimulation, but differs from increases seen with PDE5A inhibition.

To explore differential signalling from each PDE inhibitor further, RNCMs were infected with luciferase reporter plasmids to assess the signals upstream of calcineurin–NFAT, transduces myofibroblast transformation and hypertrophy, and is directly inhibited by PKG16,17. Cyclic GMP can also suppress cAMP by activating PDE28 to blunt hypertrophy and fibrosis; however cAMP rose similarly in both groups after TAC (Extended Data Fig. 6).

Figure 3 | Pressure-overload-induced cardiac pathobiology is suppressed in Pde9a−/− mice. a, Echocardiography of littermate control and Pde9a−/− left ventricle subjected to TAC. WT, wild type. b, Summary data for fractional shortening (FS), left ventricular end-systolic dimension (LV-ESD), and left ventricular mass. *P < 0.01 vs. wild-type TAC. c, Fibrosis and myocyte enlargement in sham and TAC wild-type and Pde9a−/− mice. *P < 0.01 vs. sham wild type, #P < 0.05 vs. TAC wild type, *P < 0.01 vs. TAC wild type. Scale bar, 50 μm. MT, Masson’s trichrome; WGA, wheat germ agglutinin; CSA, cross-sectional area. d, Heart and lung weight normalized to tibia length (TL). *P < 0.01 vs. sham wild type, #P < 0.05, $P < 0.01 vs. TAC wild type. e, Myocardial cGMP after TAC. *P < 0.05 vs. sham control, $P < 0.05 vs. TAC wild type. f, g, Nppa, β-myosin heavy chain (Myh7), connective tissue growth factor (Ctgf), fibronectin (Fn1), collagen type-1a (Col1a2), metallo-proteinase 2 (Mmp2), and transient receptor potential canonical 6 (Trpc6) expression, all normalized to GAPDH. *P < 0.001 vs. sham wild type, *P < 0.05 vs. TAC wild type, $P < 0.001. Data are mean ± s.e.m. and biological replicates are indicated (in parentheses for b, d-g).
activation of transcription factors known to regulate cardiac growth/ hypertrophy and survival. NFAT, MEF2 and CREB all increased with phenylephrine stimulation. NFAT declined with PDE5A-I or PDE9A inhibition, consistent with results for TRPC6 (Fig. 4e and Extended Data Fig. 8) and studies showing that both natriuretic peptide and nitric oxide stimulation block this pathway\textsuperscript{16,17}. MEF2 only declined with PDE5A-I while both GATA4 and CREB rose only with PDE9A-I (Fig. 4e). The latter two are linked to natriuretic peptide /cGMP signalling, enhanced survival and adaptive myocardial stress responses\textsuperscript{19,20}.

Lastly, we performed unbiased proteomic analysis to detect serine/ threonine phosphorylation increases in myocyte proteins altered by PDE9A-1 or PDE5A-I, with or without concomitant L-NAME. The majority (85%) of phosphorylated amino acid residues were modified by one or the other PDE inhibitor and the rest by both (Fig. 4f and Supplementary Table 1). Of these residues, L-NAME reduced 21% of those specific to PDE5A-I versus 5.2% to PDE9A-I (red symbols, \(P < 0.02\), supporting targeting of PDE9A to non-nitric-oxide-dependent cGMP (Supplementary Table 2).

The efficacy of inhibiting a cGMP-PDE to counter myocardial responses to pathological stress requires having sufficient cGMP synthesized and PDE expressed, and pathogenic signalling suppressible by PKG. Though prior work indicated PDE5A might fulfil these criteria, its preferred targeting to NOS-dependent cGMP is a potential limitation, as this pool is often depressed in cardiovascular disease. We have now identified PDE9A-I as an alternative that, unlike PDE5A-I, remains effective even when NOS-dependent cGMP synthesis is suppressed. This non-redundant function is consistent with intracellular compartmentation\textsuperscript{21}. The revelation that PDE9A serves as a natriuretic-peptide-cGMP-targeted PDE is important as this source of cGMP often rises in heart disease, whereas the NOS-derived pool declines. As with PDE5A, there are multiple downstream targets stemming from PDE9A regulation that collectively impact myocardial biology and disease. The current data identifies transcriptional controllers and protein substrates, setting the stage for future work.

The observation of heightened PDE9A expression in human heart failure, particularly HFPEF is exciting. Morbidity and mortality from
HFPEF is high and with still no effective therapies this remains a major unmet medical need worldwide.12 While there is considerable enthusiasm for CGMP-PKG-targeted treatment for this disease, recent data from a multicentre clinical trial using PDE5A-I was disappointing.13 Among potential reasons are a lack of PDE5A upregulation and low myocardial cGMP attributed to depressed nitric oxide signalling.24 Our results suggest PDE9A-I as an attractive alternative. The recent success of a combined angiotensin receptor blocker and neprilysin inhibitor25 (the latter blunting natriuretic peptide proteolysis) that is being tested in HFPEF (NCT01920711), and advances in synthetic natriuretic peptide therapies26 offer opportunities for combined treatment. PDE9A inhibitors appear well tolerated in humans and are being studied for neurocognitive disease (https://clinicaltrials.gov/ct2/show/NCT00930059). The current results support exploring these agents as new avenues for treatment of the heart, and potentially other organs in which PDE9A and the natriuretic peptide signalling system have a role.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

Received 26 March 2014; accepted 16 February 2015.

Published online 18 March; corrected online 25 March 2015 (see full-text HTML version for details).

1. Takimoto, E. et al. Chronic inhibition of cyclic GMP phosphodiesterase 9A prevents and reverses cardiac hypertrophy. Nature Med. 11, 214–222 (2005).
2. Kukreja, R. C., Salloum, F. N. & Das, A. Cyclic guanosine monophosphate signaling and phosphodiesterase-5 inhibitors in cardioprotection. J. Am. Coll. Cardiol. 59, 1921–1927 (2012).
3. Mullerhasen, F., Russwurm, M., Koelsing, D. & Friebe, A. In vivo reconstitution of the negative feedback in nitric oxide/cGMP signaling: role of phosphodiesterase type 5 phosphorylation. Mol. Biol. Cell 15, 4023–4030 (2004).
4. Castro, L. R., Verde, I., Cooper, D. M. & Fischmeister, R. Cyclic guanosine monophosphate phosphodiesterase in rat cardiac myocytes. Circulation 113, 2221–2228 (2006).
5. Carnicer, R., Crabtree, M. J., Svakumaran, V., Casadei, B. & Kass, D. A. Nitric oxide synthases in heart failure. Antioxid. Redox Signal. 18, 1078–1099 (2013).
6. Soderling, S. H., Bayuga, S. J. & Beavo, J. A. Identification and characterization of a novel family of cyclic nucleotide phosphodiesterases. J. Biol. Chem. 273, 15553–15558 (1998).
7. Fisher, D. A., Smith, J. F., Pillar, J. S., Denis, S. H. & Cheng, J. B. Isolation and characterization of PDE9A, a novel human CGMP-specific phosphodiesterase. J. Biol. Chem. 273, 15559–15564 (1998).
8. Conti, M. & Beavo, J. Biochemistry and physiology of cyclic nucleotide phosphodiesterases: essential components in cyclic nucleotide signaling. Annu. Rev. Biochem. 76, 481–511 (2007).
9. Kramer, R. J. et al. Phosphodiesterase 9A regulates central cGMP and modulates responses to cholinergic and monoaminergic perturbation in vivo. J. Pharmacol. Exp. Ther. 341, 396–409 (2012).
10. Beckman, P. R., Wouters, C. & Prickaerts, J. Phosphodiesterase inhibitors as a target for cognition enhancement in aging and Alzheimer’s disease: a translational overview. Curr. Pharm. Des. 21, 317–331 (2015).
11. Sharma, K. & Kass, D. A. Heart failure with preserved ejection fraction: mechanisms, clinical features, and therapies. Circ. Res. 115, 79–96 (2014).
12. Nausch, L. W., Ledoux, J., Bonev, A. D., Nelson, M. T. & Dostmann, W. R. Differential patterning of cGMP in vascular smooth muscle cells revealed by single GFP-linked biosensors. Proc. Natl Acad. Sci. USA 105, 365–370 (2008).
13. Guk, K., Kuc, R. E., Maguire, J. J., Fidock, M. & Davenport, A. P. Novel snake venom ligand dendroaspis natriuretic peptide is selective for natriuretic peptide receptor-A in human heart: downregulation of natriuretic peptide receptor-A in heart failure. Circ. Res. 99, 183–190 (2006).
14. Davis, J., Burr, A. R., Davis, G. F., Birnbaumer, L. & Molkentin, J. D. A TRPC6-dependent pathway for myofibroblast transdifferentiation and wound healing in vivo. Dev. Cell 23, 705–715 (2012).
15. Koitabashi, N. et al. Cyclic GMP/PKG-dependent inhibition of TRPC6 channel activity and expression negatively regulates cardiomyocyte NFAF activation; novel mechanism of cardiac stress modulation by PDE5 inhibition. J. Mol. Cell. Cardiol. 48, 713–724 (2010).
16. Kinoshita, H. et al. Inhibition of TRPC6 channel activity contributes to the antihypertrophic effects of natriuretic peptides-guanylyl cyclase-A signaling in the heart. Circ. Res. 106, 1849–1860 (2010).
17. Stangerlin, A. et al. CGMP signals modulate cAMP levels in a compartment-specific manner to regulate catecholamine-dependent signaling in cardiac myocytes. Circ. Res. 108, 929–939 (2011).
18. Piz, R. B. & Broderick, K. E. Role of cyclic GMP in gene regulation. Front. Biosci. 10, 1239–1268 (2005).
19. Oka, T. et al. Cardiac-specific deletion of Gata4 reveals its requirement for hypertrophy, compensation, and myocyte viability. Circ. Res. 98, 837–845 (2006).
20. Maurice, D. H. et al. Advances in targeting cyclic nucleotide phosphodiesterases. Nature Rev. Drug Discov. 13, 290–314 (2014).
21. Greene, S. J. et al. The CGMP signaling pathway as a therapeutic target in heart failure with preserved ejection fraction. J. Am. Heart Assoc. 2, e000536 (2013).
22. Redfield, M. M. et al. Effect of phosphodiesterase-5 inhibition on exercise capacity and clinical status in heart failure with preserved ejection fraction: a randomized clinical trial. J. Am. Med. Assoc. 309, 1268–1277 (2013).
23. van Heerebeek, L. et al. Low myocardial protein kinase G activity in heart failure with preserved ejection fraction. Circulation 126, 830–839 (2012).
24. McMurray, J. J. et al. Angiotensin-nephrilysin inhibition versus enalapril in heart failure. N. Engl. J. Med. 993–1004 (2014).
25. Zakeri, R. & Burnett, J. C. Designer natriuretic peptides: a vision for the future of heart failure therapeutics. Can. J. Physiol. Pharmacol. 89, 593–601 (2011).

Supplementary Information is available in the online version of the paper.

Acknowledgements We thank students R. D. Wardlow and X. Hu for their assistance with some of the assays and studies. This research was supported by: the National Institutes of Health (NIH) (HL-119012, HL-089297, HL-07227), Fondation Leducq TransAtlantic Network of Excellence, The Peter Belfer Foundation, Abraham and Virginia Weiss Professorship (D.A.K.); HL-093432 (E.T.), American Heart Association (D.I.L.) and Max Kade Fellowship of the Austrian Academy of Sciences (P.P.R.). Proc. Natl Acad. Sci. USA 110, 2221–2228 (2013).

We thank students R. D. Wardlow and X. Hu for their assistance with some of the assays and studies. This research was supported by: the National Institutes of Health (NIH) (HL-119012, HL-089297, HL-07227), Fondation Leducq TransAtlantic Network of Excellence, The Peter Belfer Foundation, Abraham and Virginia Weiss Professorship (D.A.K.); HL-093432 (E.T.), American Heart Association (D.I.L.) and Max Kade Fellowship of the Austrian Academy of Sciences (P.P.R.). Proc. Natl Acad. Sci. USA 110, 2221–2228 (2013).

We thank students R. D. Wardlow and X. Hu for their assistance with some of the assays and studies. This research was supported by: the National Institutes of Health (NIH) (HL-119012, HL-089297, HL-07227), Fondation Leducq TransAtlantic Network of Excellence, The Peter Belfer Foundation, Abraham and Virginia Weiss Professorship (D.A.K.); HL-093432 (E.T.), American Heart Association (D.I.L.) and Max Kade Fellowship of the Austrian Academy of Sciences (P.P.R.). Proc. Natl Acad. Sci. USA 110, 2221–2228 (2013).

We thank students R. D. Wardlow and X. Hu for their assistance with some of the assays and studies. This research was supported by: the National Institutes of Health (NIH) (HL-119012, HL-089297, HL-07227), Fondation Leducq TransAtlantic Network of Excellence, The Peter Belfer Foundation, Abraham and Virginia Weiss Professorship (D.A.K.); HL-093432 (E.T.), American Heart Association (D.I.L.) and Max Kade Fellowship of the Austrian Academy of Sciences (P.P.R.). Proc. Natl Acad. Sci. USA 110, 2221–2228 (2013).

We thank students R. D. Wardlow and X. Hu for their assistance with some of the assays and studies. This research was supported by: the National Institutes of Health (NIH) (HL-119012, HL-089297, HL-07227), Fondation Leducq TransAtlantic Network of Excellence, The Peter Belfer Foundation, Abraham and Virginia Weiss Professorship (D.A.K.); HL-093432 (E.T.), American Heart Association (D.I.L.) and Max Kade Fellowship of the Austrian Academy of Sciences (P.P.R.). Proc. Natl Acad. Sci. USA 110, 2221–2228 (2013).
METHODS

Human myocardial tissue. Procurement of human myocardial tissue was performed under protocols approved by Institutional Review Boards at the University of Pennsylvania (Pennsylvania, USA), Johns Hopkins University (Maryland, USA) and VU University Medical Center, (Amsterdam, The Netherlands) and its coordinated affiliated centres and consent for biopsy procedures or use of explanted tissues prospectively obtained in all cases. Explant dilated non-ischaeimic failing human hearts were procured at the time of orthotopic heart transplantation at the Hospital of University of Pennsylvania. Non-failing hearts were obtained at the time of organ donation from cadaveric donors. In all cases, hearts were arrested in situ using ice-cold cardioplegia solution, transported on wet ice, and flash frozen in liquid nitrogen within 4 hours of explantation. All samples were thawed, thickness biopsies obtained from the free wall of the left ventricle. HFPEF patients were referred for cardiac catheterization and left ventricular endomyocardial biopsy because of clinical suspicion of restrictive cardiomyopathy. Left ventricular biopsies were procured using femoral artery access and a long biopsy gun. Diagnostic criteria and clinical characteristics of the HFPEF and aortic stenosis patients have been previously reported. Control samples for these studies were obtained from explanted unused donor hearts.

Pde9a knockout mouse. All protocols involving animals followed US National Institutes of Health guidelines and were approved by the animal and care use committee of the Johns Hopkins Medical Institutions. Pde9a global knockout (Pde9a−/−) mice were developed by Pfizer Inc. The model replaced exon 12 in the catalytic domain of Pde9a with a lacZ-neomycin cassette (see Extended Data Fig. 1). Mice did not express any splice variants as they all share this sequence. Pde9a−/− mice were born in normal Mendelian ratios, and had no evident physiological or behavioural abnormalities (Supplementary Table 3). Expression of alternative cGMP-targeting PDEs, PDE1A and PDE5A were not significantly different in Pde9a−/− versus littermate control hearts (Extended Data Fig. 9).

Transverse aortic constriction and chronic drug studies. Pressure overload was performed by surgical placement of suture around the transverse aorta sized to the 27G needle, as described previously. For chronic drug treatment studies, size, age- and sex-matched (male) C57BL/6J mice (Jackson Labs) were randomized to receive vehicle, PF-9613 (30 mg kg−1 per day with Bioserv soft diet). The mean free plasma concentration of PF-9613 was 77 nM, (peak of 1.5 μM PF-04449613 inhibited 70% of PDE9A without altering PDE5A activity). Fltn1 (Mm01255770_g1), Nppa (Mm01255777_g1), Nppb (Mm01255770_g1), Mmp2 (Mm00501049_m1), Pde9a (Mm00483888_m1), Ctgf (Mm01192993_g1), Fn1 (Mm01256744_m1), Gapdh (Mm00999995_g1), Myh7 (Mm00600555_m1), Nppa (Mm01255777_g1), Nppb (Mm01255770_g1), Mmp2 (Mm00434998_m1), Pde9a (Mm00501049_m1), Pde5a (Mm00461777_m1), Trpc6 (Mm01176083_m1). For SYBR primers used: rat Nppa (forward 5′-ATACAGTGGCGTGTTCAACACAGA-3′, reverse 5′-TGCACCTCATCTGTCGCCGACT-3′), rat Nppb (forward 5′-ATCGAGAAGCTGTGGATGGTTAAGTA-3′, reverse 5′-CTTCTGGCGGCAAAGCAGCTGGAACCT-3′), rat Gapdh (forward 5′-GACATGCCGCAGGATGGAAC-3′, reverse 5′-GAGCCAGAGGTCCCTTATTCG-3′), rat Pde9a (forward 5′-AACCTGTTGGAACACTTTTCTTGTTCTGTTTG-3′, reverse 5′-TTGTTGTTGATGCCTTGCCAAGGAAA-3′). All PCR samples were run in duplicate and normalized to GAPDH. Specificity of the SYBR green assays was confirmed by dissociation curve analysis.

Protein electrophoresis and immunoblot assays followed standard procedures, using lysis buffer (Cell Signaling) with protease inhibitor PMSF (1 mM), and run on NuPAGE 4 to 12% gel (Invitrogen). Membranes were probed for human anti-PDE9A (1:1,000), mouse anti-GAPDH or human anti-actin (1:10,000, Cell Signaling). Tissue expression using standard procedures. PCR primers were: TaqMan primers used: COL1A2 (Mm00430081_m1), COL3A1 (Mm00439294_m1), FN1 (Mm00483551_m1), LGALS3 (Mm00463629_m1), LAMB3 (Mm00439070_m1), LIMK1 (Mm00433618_m1), SFRP4 (Mm00435276_m1), TAC3 (Mm00430202_m1), TAC4 (Mm00430197_m1), MUC5AC (Mm00483970_m1), MUC7 (Mm00504285_m1), MUC12 (Mm00504285_m1), MUC14 (Mm00504285_m1), MUC16 (Mm00504285_m1). For SYBR primers used: rat Nppa (forward 5′-ATACAGTGGCGTGTTCAACACAGA-3′, reverse 5′-TGCACCTCATCTGTCGCCGACT-3′), rat Nppb (forward 5′-ATCGAGAAGCTGTGGATGGTTAAGTA-3′, reverse 5′-CTTCTGGCGGCAAAGCAGCTGGAACCT-3′), rat Gapdh (forward 5′-GACATGCCGCAGGATGGAAC-3′, reverse 5′-GAGCCAGAGGTCCCTTATTCG-3′), rat Pde9a (forward 5′-AACCTGTTGGAACACTTTTCTTGTTCTGTTTG-3′, reverse 5′-TTGTTGTTGATGCCTTGCCAAGGAAA-3′). All PCR samples were run in duplicate and normalized to GAPDH. Specificity of the SYBR green assays was confirmed by dissociation curve analysis.

Real-time myocyte cGMP analysis. RCNMs plated on gelatin-coated 35-mm glass-bottom dishes and collected cGMP responses to forskolin (0.1 μM) and 8-BrcGMP (1 μM) in the presence or absence of a selective inhibitor of PDE9A (1 μM) after a 20-min equilibration and 30 min of cGMP accumulation. Fluorescence was acquired using a 63× oil immersion objective (N.A. 1.4) on a Leica TCS SP5 confocal microscope. Image acquisition was performed on a Zeiss LSM510-META laser scanning microscope.

Luciferase reporter assay. RCNMs were transfected with Nfat-luc, Creb1-luc, Gata4-luc, TK-luc (Promega) or Myf-2-luc (Addgene), using Xfect reagent (Clontech) following manufacturer's instructions. Co-transfection of an adenovirus expressing FlincG13 (courtesy of W.R.D.) at a multiplicity of infection of 10, for 24–48 h at 37 °C, 5% CO2 until a 90% transfection efficiency was achieved. Since the absolute level of expressed FlincG varied among cells, each cell was used as their own control, with stimulation protocols compared within a given cell by paired analysis. Cell imaging was performed in imaging buffer (Hank’s balanced salt solution (HBSS), Mediatech, Inc.) by using a 3× spinning disk confocal system on a Zeiss microscope with SlideBook 5.0 software (Intelligent Imaging Innovations, Inc.), a 63× oil dipping objective (N.A. 1.0), and Ixon ENCCD DVB camera with 1-s acquisitions and 20 ms exposure time, exciting with a solid state laser at 488 nm and collecting the emission at 510 nm at 37 °C. cGMP responses were assessed by measuring the emission fluorescence (expressing FlincG13) at a sensitivity of 0.5. All data were acquired and analyzed using an algorithm with NIH Image J 1.47i software. Image acquisition was performed on a Zeiss LSM510META laser scanning microscope.
to the manufacturer’s protocol. After 24 h transfection, cells were stimulated with 25 μM of phenylephrine for 6 h, lysates harvested using passive lysis buffer (Promega) and luciferase activity determined using the Dual-Luciferase Reporter Assay System (Promega) and Veritas 96-well microplate luminometer (Turner Biosystems) following the manufacturer’s protocol.

**Phosphoproteomic analysis.** Samples (n = 4 per group) were lysed in 8 M urea, 0.5% SDS with brief sonication and protein concentration determination by the BCA method. For each sample, 200 μg of total protein was digested with trypsin/ Lys-C protease mixture (Promega) according to previously published methods30, samples desalted on 10-mg Oasis HLB cartridges (Waters) and eluted in 300 μl of 80% acetonitrile, 5% trifluoroacetic acid and 1 M glycolic acid, and enriched by titanium dioxide (TiO2). Enriched peptides were desalted as above but eluted in 200 μl of 80% ACN, 0.1% formic acid and dried under vacuum. Dried peptides were re-suspended in 20 μl of 0.1% FA for liquid chromatography–tandem mass spectrometry analysis.

For each sample, 4 μl was injected in duplicate onto an EASY-nLC 1000 (mobile phase A was 0.1% FA in water and mobile phase B was 0.1% FA in ACN) connected to a Q-Exactive Plus (Thermo) equipped with a nano-electrospray ion source. All raw MS/MS data was searched using the Sorcerer 2TM-SEQUEST algorithm (Sage-N Research) using default peak extraction parameters. Post-search analysis was performed using Scaffold 4 (Proteome Software, Inc.) with protein and peptide probability thresholds set to 95% and 90%, respectively, and one peptide required for identification, and these the spectra were manually validated. Phosphosite localization was determined using Scaffold PTM version 2.1.3 and phosphosites with probabilities less than 90% were ignored. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD001585.

**Statistics and reproducibility.** For all analyses, sample size is reported in the figure legends or figures themselves. Most in vitro studies were done with two to three sets of independent experiments. Comparisons of multiple groups were performed using either one-way or two-way ANOVA (as appropriate). If normality or equal variance tests failed, then a Kruskal–Wallis test was used. Two-group analysis used either a Student’s t-test or a non-parametric Mann–Whitney test. Post-hoc multiple comparisons testing used either a Tukey or Dunn’s test. Analysis of time-dependent changes was performed by ANCOVA with repeated measures. Formal power analysis was not prospectively performed, though for variables where variance was known we could estimate sample size based on an anticipated mean effect.

27. Seo, K. et al. Combined TRPC3 and TRPC6 blockade by selective small-molecule or genetic deletion inhibits pathological cardiac hypertrophy. Proc. Natl Acad. Sci. USA 111, 1551–1556 (2014).
28. Verhoest, P. R. et al. Design and discovery of 6-[(3S,4S)-4-methyl-1-(pyrimidin-2-ylmethyl)pyrrolidin-3-yl]-1-(tetrahydro-2H-pyran-4-yl)-1,5-dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (PF-04447943), a selective brain penetrant PDE9A inhibitor for the treatment of cognitive disorders. J. Med. Chem. 55, 9045–9054 (2012).
29. Zhou, B. et al. Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. Nature 454, 109–113 (2008).
30. Kirk, J. A. et al. Cardiac resynchronization sensitizes the sarcomere to calcium by reactivating GSK-3beta. J. Clin. Invest. 124, 129–139 (2014).
Extended Data Figure 1 | Development of Pde9a knockout (Pde9a<sup>−/-</sup>) and specificity of Pde5a or Pde9a siRNA. a, Pde9a knockout (Pde9a<sup>−/-</sup>) mice were developed by replacing the exon 12 region with lacZ-neomycin cassette in the catalytic domain of the carboxy terminal in the Pde9a gene. The genotyping was performed using specific primers designed between exons 11 and 13 including neomycin as following: Gs1 (5'-CACAGATGATACAGTATGGTCTGG-3'), Gs2 (5'-TGCAGTCATCAGGACCAAGATGTCC-3'), and Neo (5'-GACGAGTTCTTCTGAGGGGATCGATC-3'). b, The typical genotyping pattern of Pde9a<sup>−/-</sup> mice was shown on 2% agarose gel (250 bp for wild type and 500 bp for Pde9a<sup>−/-</sup> mice). c, Selective gene silencing using siRNAs targeting PDE5A or PDE9A. PCR confirms specificity and substantial gene knockdown achieved in cell culture (n = 6 per group).
Extended Data Figure 2 | Expression of PDE9A protein in RNCM, mouse brain and human heart. a, Immunoblot for PDE9A in neonatal cardiomyocytes transfected with either scrambled control siRNA (Sc) or Pde9a siRNA (siR), confirming the suppression of protein expression by the siRNA. A control gel for comparison was derived from brain tissue using Pde9a−/− mice and littermate controls. The band identified at 60 to 65 kDa was similar in both tissues. PDE9A bands are usually identified between 55–70 kDa depending on the splice variants expressed in a given tissue and species. b, Control immunohistochemistry showing that PDE9A detected by antibody can be largely quenched (inactivated) selectively by preincubation with recombinant ligand; scale bar, 50 μm. c, Immunostaining of PDE9A from all 8 control and DCM patients; scale bar, 200 μm. There was consistent enhanced staining in DCM patients versus controls. All images were obtained at an identical level of laser illumination and have not been altered.
Extended Data Figure 3 | Selectivity of PDE9A inhibitors. a. The dose responses of recombinant PDE9A or PDE5A to a selective PDE9A inhibitor (PF-9613) and sildenafil. Data performed in triplicate at each point. A dose of 5 μM PF-9613 inhibited PDE9A effectively, but had negligible impact on PDE5A. By contrast, the PDE5A inhibitor sildenafil inhibited PDE5A by 80% at a dose of 1 μM, commonly used for cells and tissue, but had no impact on PDE9A at this dose. These doses were therefore used in our cell-based studies.

b. Confirmation that an alternative PDE9A inhibitor (PF-04447943), currently being tested in humans, shows similar anti-hypertrophic effects as PF-9613 in cardiac myocytes (n = 4 per group); *P < 0.001 vs. baseline; #P < 0.01 vs. phenylephrine. c. Inhibition of PKG activity with DT3 reverses the suppression of phenylephrine-stimulated Nppb gene expression by PF-9613. This is a companion panel to Fig. 2b, bottom. n = 6 for basal (no drugs), n = 8 for other groups; *P < 0.001 vs. baseline; #P < 0.01 vs. phenylephrine. Data are mean ± s.e.m.
Extended Data Figure 4 | PKG activity or cGMP measurement of ANP with inhibitors in RNCM. a, PF-9613 significantly increases PKG activity assessed by in vitro assay upon stimulation with ANP; \( n = 4 \) per group. \( *P < 0.01 \) vs. other groups. b, PF-9613 augmentation of ANP-stimulated cGMP is not altered due to gene silencing of Pde5a; \( n = 5 \). This differs from the complete suppression of cGMP modulation by PF-9613 in myocytes with genetically silenced Pde9a (Fig. 1g). c, SIL does not enhance cGMP stimulated by ANP. This contrasts to its augmentation of nitric-oxide-donor-derived cGMP (Fig. 1h); \( n = 4 \). \( *P < 0.01 \) vs. basal state, \#\( P < 0.01 \) vs. ANP. Data are mean ± s.e.m.
Extended Data Figure 5 | Confocal immunostaining of cardiomyocyte PDE9A and PDE5A. a, PDE9A does not co-localize with α-actinin at the Z-band, whereas PDE5A does. b, PDE9A does not co-localize with α-actinin in rat neonatal myocytes. c, PDE9A co-localizes with T-tubular membranes as defined by antibody staining against the sarcoplasmic reticular ATPase-2 (SERCA2a). This differed from the localization of PDE5A. Scale bars, 20 μm.
Extended Data Figure 6 | Myocardial cAMP levels in controls (sham wild type) and Pde9a<sup>−/−</sup> mice before and after TAC. The cAMP levels were increased in TAC wild type, but they were not affected by modulation of PDE9A expression. Sham wild type, n = 4; TAC wild type, n = 5; sham Pde9a<sup>−/−</sup>, n = 6; TAC Pde9a<sup>−/−</sup>, n = 10. Data are mean ± s.e.m.
Extended Data Figure 7 | Effect of PF-9613 on blood pressure and cardiac function in mouse.  

a, Acute administration of PF-9613 by gavage was studied to assess effects on cardiac pressures, and contractility (end-systolic elastance; Ees). Over a 1-h observation period (peak plasma concentrations found after 30 min) there was no change in any of these parameters; n = 3.

b, Chronic treatment of sham control mice with PF-9613 for 3 weeks (n = 3) revealed no effect on cardiac function, mass or volumes. EF, ejection fraction; FS, fractional shortening; LV-ESD, left ventricular end-systolic cross-sectional dimension; LV-EDD, left ventricular end-diastolic cross-sectional dimension. Data are mean ±s.e.m.
Extended Data Figure 8 | Effect of chronic PDE9A inhibition on left ventricular mass, lung weight and alteration of TAC-responsive genes.

a, Post-mortem analysis of heart mass and lung weight (both normalized to tibia length) from mice subjected to 5 weeks of pressure overload (TAC) and co-treated with either a vehicle control, PDE9A inhibitor or PDE5A inhibitor. A sham-operation control group is also shown; sham, n = 6; TAC, n = 9; TAC + PF9613, n = 9; TAC + SIL, n = 5. 

b, Molecular analysis of TAC-responsive (increased expression) genes, including showing similar reductions from either PDE inhibitor in some (for example, Trpc6), a disparity between inhibitors with significant or borderline greater efficacy from PDE9A inhibition in others (for example, Ctgf, Nppa, *P* < 0.02 and *P* < 0.1, respectively between PDE5A and PDE9A inhibitor response), and substantial disparities in others (for example, Fn1, *P* < 0.001 between PDE5A and PDE9A inhibition). Sham, n = 5; TAC, n = 5; TAC + PF9613, n = 6; TAC + SIL, n = 5. *P* < 0.01 vs. sham; †*P* < 0.001 vs. sham; #*P* ≤ 0.05; ††*P* < 0.01; §§*P* < 0.001 vs. TAC. Data are mean ± s.e.m.
Extended Data Figure 9 | Gene expression of cGMP-hydrolyzing PDEs in Pde9a−/− and littermate controls. n = 10 per group. The mouse model deleted Pde9a gene expression (normalized to Gapdh), but did not impact the expression of the two other cGMP-regulating PDEs in mouse: Pde1a or Pde5a.
**Extended Data Table 1 | Clinical characteristics of dilated heart failure and donor control patients**

|                          | Non-Failing Controls | Dilated HF   | p     |
|--------------------------|----------------------|-------------|-------|
| Heart Weight/Body Weight (g/kg) | 4.9 ± 0.6            | 7.1 ± 1.0   | <0.0001 |
| Ejection Fraction (%)    | 53.1 ± 2.0           | 14.3 ± 4.2  | <0.0001 |
| Biventricular Pacing (CRT) | 0                   | 70%         | 0.003  |
| History of VT/VF         | 0                    | 60%         | 0.01   |
| History of Diabetes      | 30%                  | 30%         | NS     |
| Drugs:                   |                      |             |       |
| Amiodarone               | 0%                   | 60%         | 0.01   |
| Beta-blockers            | 0%                   | 90%         | <0.001 |
| ACE-inhibitors           | 40%                  | 20%         | NS     |
| Nitrates                 | 0%                   | 30%         | NS     |

CRT, cardiac resynchronization therapy; VT, ventricular tachycardia; VF, ventricular fibrillation.