BRIEF ULTRARAPID COMMUNICATION

Microdomain Switch of cGMP-Regulated Phosphodiesterases Leads to ANP-Induced Augmentation of β-Adrenoceptor-Stimulated Contractility in Early Cardiac Hypertrophy

Ruwan K. Perera[1,2,3], Julia U. Sprenger[1,2,3], Julia H. Steinbrecher[2], Daniela Hübscher[1,2], Stephan E. Lehnart[2,4] , Marco Abesser[5], Kai Schuh[5], Ali El-Armouche[2,6] and Viacheslav O. Nikolaev,[1,2,3,4]

[1]Emmy Noether Group of the DFG, European Heart Research Institute Göttingen; [2]Heart Research Center Göttingen, Georg August University Medical Center, D-37075 Göttingen, Germany; [3]Institute of Experimental Cardiovascular Research, University Medical Center Hamburg-Eppendorf, D-20246 Hamburg, Germany; [4]German Center for Cardiovascular Research (DZHK); [5]Instutite of Physiology, University of Würzburg, D-97070 Würzburg, Germany, and; [6]Institute of Pharmacology and Toxicology, Dresden University of Technology, D-01307 Dresden, Germany.

Running title: Molecular Basis of Hypercontractility

Subject codes:
[145] Genetically altered mice
[138] Cell signaling/signal transduction
[108] Other myocardial biology

Address correspondence to:
Dr. Viacheslav O. Nikolaev
Institute of Experimental Cardiovascular Research
University Medical Center Hamburg-Eppendorf
Martinistr. 52
D-20246 Hamburg
Germany
Tel: +49-40-7410-51391
Fax: +49-40-7410-40180
v.nikolaev@uke.de

In January 2015, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 14.7 days.

DOI: 10.1161/CIRCRESAHA.116.306082

ABSTRACT

***Rationale*:** Cyclic nucleotides are second messengers that regulate cardiomyocyte function through compartmentalized signaling in discrete subcellular microdomains. However, the role of different microdomains and their changes in cardiac disease are not well understood.

Objective: To directly visualize alterations in β-adrenergic receptor (β-AR)-associated 3',5'-cyclic adenosine (cAMP) and guanosine (cGMP) microdomain signaling in early cardiac disease.

***Methods and Results*:** Unexpectedly, measurements of cell shortening revealed augmented β-AR-stimulated cardiomyocyte contractility by atrial natriuretic peptide (ANP)/cGMP signaling in early in cardiac hypertrophy following transverse aortic constriction, which was in sharp contrast to well-documented β-adrenergic and natriuretic peptide signaling desensitization during chronic disease. Real-time cAMP analysis in β_1- and β_2-AR-associated membrane microdomains using a novel membrane-targeted Förster resonance energy transfer-based biosensor transgenically expressed in mice revealed that this unexpected ANP effect is brought about by spatial redistribution of cGMP-sensitive phosphodiesterases 2 and 3 between both receptor compartments. Functionally, this led to a significant shift in cGMP/cAMP cross-talk and in particular, to cGMP-driven augmentation of contractility in vitro and in vivo.

***Conclusions*:** Redistribution of cGMP-regulated phosphodiesterases and functional reorganization of receptor-associated microdomains occurs in early cardiac hypertrophy, affects cGMP-mediated contractility and might represent a previously not recognized therapeutically relevant compensatory mechanism to sustain normal heart function.

Keywords:
cAMP, cGMP, ANP, microdomains, phosphodiesterase inhibitor, hypertrophy, cardiac dysfunction, cardiomyocyte hypertrophy, cyclic nucleotide, adrenergic receptor.

Nonstandard Abbreviations and Acronyms:

cAMP	3',5'-cyclic adenosine monophosphate
ANP	atrial natriuretic peptide
β-AR	β-adrenergic receptor
cGMP	3',5'-cyclic guanosine monophosphate
FRET	Förster resonance energy transfer
IBMX	3-isobutyl-1-methylxanthine
ISO	isoproterenol
αMHC	α-myosin heavy chain
PDE	phosphodiesterase

INTRODUCTION

The cyclic nucleotides 3',5'-cyclic adenosine (cAMP) and guanosine monophosphates (cGMP) are ubiquitous second messengers which regulate cardiomyocyte function by acting in discrete subcellular microdomains. In particular, it has been proposed that such microdomains are based on differentially localized receptors, cAMP-dependent protein kinases (PKA), its anchoring proteins, and cAMP/cGMP hydrolyzing enzymes phosphodiesterases (PDEs) involved in termination and spatial confinement of cyclic nucleotide signaling.[1,2] cGMP can regulate compartmentalized cAMP signaling by either stimulating PDE2 or inhibiting PDE3 activity.[3,4] However, due to the lack of real-time imaging techniques, cyclic nucleotide dynamics in distinct functionally relevant microdomains of adult cardiomyocytes and their role in disease are not well understood.

Two major subtypes of cardiac α-adrenergic receptors αARs) modulate the effects of catecholamines on excitation-contraction coupling. The predominantly G_s-coupled β_1-AR is evenly distributed across the sarcolemma and stimulates far-reaching cAMP signals, which strongly increase contractility and promote cardiac remodelling. In contrast, the β_2-AR couples to both G_s- and G_i-proteins and is exclusively located in transverse (T)-tubules, thereby generating highly localized cAMP responses which may protect from apoptosis and pathological remodelling.[5,6] cGMP has been reported as a "cardioprotective" second messenger, since stimulation of cGMP production or inhibition of its degradation by cGMP-PDE inhibitors leads to a reduction of β-AR-stimulated pathological hypertrophy.[7] Conversely, genetic deletion of the atrial/brain natriuretic peptide (ANP/BNP) receptor guanylyl cyclase A (GC-A) promotes cardiac hypertrophy.[8,9] One well-established hallmark of chronic cardiac disease is the dramatic reduction of β_1-AR densities,[10] its desensitization and a decrease of GC-A activity.[11] However, little is known about changes in microdomain-specific cAMP/cGMP signaling in diseased cardiomyocytes, especially not at disease onset which is most relevant for therapeutic interventions.

Here, we show that during early cardiac hypertrophy and contrary to expectation, ANP augments catecholamine-stimulated force of single myocyte contraction as well as heart rates in intact hearts and in vivo. Using a novel transgenically expressed Förster resonance energy transfer (FRET)-based cAMP biosensor, we uncover that this is caused by a spatial redistribution of PDE2/3 between the β_1-and β_2-AR-associated microdomains, without any change of whole-cell PDE expression and activities. This represents a novel adaptation mechanism which may compensate early disease-driven changes and transiently support contractile function during pressure overload before the transition to decompensated chronic disease.

METHODS

Detailed methods section is available in the *Online Supplement*.

FRET-based cAMP measurements in cardiomyocytes.
Adult mouse ventricular myocytes were freshly isolated and subjected to FRET measurements as described.[12]

Transverse aortic constriction (TAC).
All animal experiments were performed according to institutional and governmental guidelines as described.[13] Mice were sacrificed 8 weeks after surgery for ventricular cardiomyocyte isolation or whole-heart measurements.

Statistics.
Normal distribution was tested by the Kolmogorov-Smirnov test. Differences were analyzed using OriginPro8.6 software (OriginLab, Northampton) and one-way ANOVA or Mann-Whitney tests, followed by Bonferroni's post-hoc test. Data are presented as means±SE from the indicated number of independent experiments (animals and cells) per condition.

RESULTS

ANP increases contractility in hypertrophied cardiomyocytes and hearts.

ANP has previously been found not to affect the isoproterenol (ISO)-stimulated contractility of single healthy mouse ventricular cardiomyocytes.[14] Accordingly, ANP did not significantly change sarcomere shortening after submaximal ISO stimulation of healthy cells (Figure 1A,C). However, in cardiomyocytes isolated from hearts after transverse aortic constriction (TAC), ANP applied after ISO but not alone strikingly led to a significant positive inotropic response (Figure 1B,C). This effect could be mimicked by a cell-permeable cGMP analogue cGMP-AMP but not by the selective NPR-C (clearance) receptor agonist cANP(4-23) (Online Figure IA), suggesting that it is modulated by the GC-A/cGMP pathway. Interestingly, the stimulatory effect of ANP was observed also in Langendorff-perfused intact TAC hearts where it could further increase the beating rate after ISO prestimulation (Figure 1D). This finding could be also confirmed by hemodynamic measurements in vivo, where ANP augmented ISO-induced increase of heart rate and showed a strong tendency for an increased cardiac output (Figure 1E,F). Remarkably, the ANP-stimulated cGMP levels were not reduced in TAC cells (Online Figure IB), suggesting the absence of GC-A desensitization. This behaviour of hypertrophied cardiomyocytes, which is in contrast to well-established β-AR and ANP receptor desensitization[11,15] and absence of ANP effect on contractility occurring in chronic heart failure[16,17] might indicate both early and specific disease-associated changes in cGMP/cAMP-dependent signaling. To study these changes in more detail, we performed real-time imaging of cAMP.

FRET imaging reveals altered subtype-specific β-AR-cAMP responses in diseased cardiomyocytes.

Since membrane receptor-associated cAMP microdomains have been shown to regulate functional contractile responses, we sought to monitor cAMP dynamics directly in specific membrane compartments. To achieve this goal, we generated a novel transgenic mouse which expresses a cAMP sensor pmEpac1 targeted to caveolin-rich sarcolemmal microdomains (Online Figure II). Importantly, the sensor transgenic mice were indistinguishable from their control littermates in terms of heart function and cell size, showing no adverse cardiac phenotype (Online Figure III). Side-by-side comparison with the previously described cytosolic otherwise identical version of the same biosensor (Epac1-camps) revealed that pmEpac1 showed slightly lower affinities to cAMP but could much better resolve local β_2-AR cAMP signals (Online Figure IV), which are known to compartmentalize close to T-tubular membranes.[6]

After TAC, pmEpac1 mice showed increased heart size and wall thickness with only slightly reduced functional parameters, indicative of a relatively mild, functionally still compensated phenotype of pathological hypertrophy, which was accompanied by a 2-fold increase of plasma ANP-levels (Online Figure V). In isolated cardiomyocytes, the localization of the FRET sensor in the striation-associated T-tubular membranes and surface sarcolemma was virtually unchanged (Online Figure VIA,B). First, we tested whether ANP has any effect on subsarcolemmal cAMP levels under the same experimental conditions as in Figure 1. We prestimulated TAC and sham myocytes with a low dose of ISO (3 nmol/L) and next applied 100 nmol/L of ANP. Indeed, ANP led to augmentation of ISO-stimulated cAMP levels

exclusively in TAC cells (Figure 2A,B). To further test how cAMP levels are affected at saturating β-AR agonist concentrations and which receptor subtypes are involved, we compared β_1- and β_2-AR specific responses in TAC vs sham cells and found significantly enhanced global ISO responses which were due to stronger β_1-AR-signals (Figure 2C,D,F). In contrast, β_2-AR-stimulated cAMP (β_2-AR-cAMP) responses were significantly reduced in diseased cells (Figure 2E,F). These changes of β-AR-cAMP signals were not due to altered β-AR densities (164±16 vs 128±17 fmol/mg membrane protein in sham vs. TAC cells, not significant, p=0.1, n=3) or β_1-/β_2-AR ratio, as confirmed by radioligand binding studies (Online Figure VIC). In contrast to TAC cardiomyocytes, cells isolated from pmEpac1 hearts with a more severe heart failure phenotype established 12 weeks after myocardial infarction showed marked downregulation of β_1-AR-cAMP signaling with unchanged β_2-AR responses (Online Figure VII).

In hypertrophy, cGMP-sensitive PDEs redistribute between β_1-AR and β_2-AR associated membrane microdomains.

We next studied the effects of various PDE inhibitors after global and β_2-AR-selective receptor stimulations in healthy cells. We found that while the β_1-AR-cAMP microdomain is mostly controlled by PDE4 (followed by less active PDE3 and PDE2), the β_2-AR-cAMP microdomain is under predominant control of PDE3, which was not detectable using the cytosolic FRET sensor Epac1-camps (Online Figure VIII). The new membrane localized pmEpac1 sensor revealed that in diseased cells, PDE2 effects after global β_1-AR stimulation were significantly reduced, while PDE3 and 4 inhibitor responses remained unchanged (Figure 3A-C). In contrast, the predominant regulation of β_2-AR-cAMP by PDE3 in this microdomain was abolished. Instead, the PDE2 effects were significantly increased, while PDE4 effects remained unchanged (Figure 3D-F). These findings suggest a redistribution of PDE2 from the β_1-AR to the β_2-AR-associated microdomains and a selective downregulation of PDE3 in the β_2-AR compartment. To test whether the disease leads to changes in physical localization of PDE2 and 3, we stained the cells with PDE family-specific antibodies and analyzed the degree of their co-localization with α-actinin. Interestingly, TAC led to measurable alterations in subcellular PDE2 and 3 distribution (Online Figure IX). Furthermore, re-localization of PDE2 between β_1-AR and β_2-AR microdomains could be observed in neonatal rat cardiomyocytes expressing affinity-tagged receptors (Online Figure X). Subcellular fractionation of sham and TAC heart revealed a movement of PDE2 from β_1-AR-associated non-caveolar into β_2-AR-containing caveolar fractions in disease, while PDE3 proportion in caveolin-positve fractions was reduced (Online Figure XI). These data directly support our hypothesis that altered PDE2/3 inhibitor effects indeed arise from subcellular re-localization of these PDEs.

Previously, it has been shown that under advanced cardiac hypertrophy, PDE3 and PDE4 activities are downregulated in rodent and human cardiomyocytes,[18,19] while PDE2 activity (which is a crucial PDE for the regulation of submembrane ANP/GC-A/cGMP pools)[20] is increased in various heart failure settings, albeit not under compensated hypertrophy.[21] Strikingly, in our compensated mouse hypertrophy model, total PDE2-4 activities were unchanged (Online Figure XIIA). Likewise, net expression levels of PDE2 analyzed by immunoblotting with whole-cell lysates were unaltered (Online Figure XIIB). In addition, protein levels of the PDE4D8 isoform, which directly interacts with β_1-AR,[22] or of β-arrestin 2, which recruits PDEs to the β_2-AR, and of the functionally-relevant IBMX-insensitive PDE8A[23] were also unaffected (Online Figure XIIC). Collectively, these data indicate an early subcellular remodelling of PDEs and a switch between two cGMP-regulated PDEs in the β_2-AR microdomain, i.e. increase in local cGMP-stimulated PDE2 and a decrease in cGMP-inhibited PDE3 activities, occurring without any change of global PDE activity and content at the cellular level. Interestingly, using immunoblot analysis we could detect a disease-associated shift of PDE3A isoforms from the primarily expressed PDE3A2 to the longest PDE3A1 isoform (Online Figure XIII), which is localized predominantly at sarcoplasmic reticulum instead of the plasma membrane[24]. Another potential mechanism of different PDE localization might be an altered

expression of scaffolding proteins[5], of which AKAP13 (AKAP-Lbc) was increased while PI3γ kinase was unchanged in our disease model (Online Figure XIII).

cGMP-dependent regulation of β_2-AR-cAMP signals is altered in hypertrophy.

To study whether the altered balance between PDE2 and PDE3 in hypertrophy contributes to the cGMP-dependent regulation of cAMP levels in the β_2-AR microdomain, we measured the effect of ANP in TAC and sham pmEpac1 cardiomyocytes after selective β_2-AR stimulation. Addition of ANP could further enhance the β_2-AR-prestimulated cAMP levels in healthy cells, which was due to cGMP-mediated PDE3 inhibition, because this effect was abolished in the presence of the PDE3-selective inhibitor cilostamide (Figure 4A,B). In diseased cardiomyocytes, the ANP effect on β_2-AR-cAMP levels was dramatically reduced. However, pretreatment of cells with the PDE2-selective inhibitor BAY-60-7550 (BAY) could fully reverse this effect (Figure 4C,D), suggesting that in cardiac hypertrophy, the redistribution of PDE2 and PDE3 inverses the cGMP-mediated effects on the β_2-AR-cAMP pool. The described cGMP dependent effects on β_2-AR-cAMP did also directly translated into cell contractile responses (Online Figure XIVA,B, Figure 4E), which were exactly the opposite to the increased contractility after ANP application in TAC cells under global β-adrenergic stimulation (described in Figure 1), further supporting the idea of PDE2 and PDE3 redistribution between receptor-associated microdomains. Interestingly, cGMP-AM, which directly acts on these redistributed PDEs could also here recapitulate the effect of ANP. Thus, the observed changes of local cAMP levels monitored by FRET are directly translated into contractile response, specifically shifting the ANP-mediated effect on β-adrenoceptor-stimulated contractility from the β_2-AR to the β_1-AR associated compartment.

DISCUSSION

In this study, we directly monitored compartmentalized effects of cAMP and cGMP on cell function in early cardiac disease and uncovered unexpected positive inotropic and chronotropic effects of ANP after β-AR stimulation. These changes are in sharp contrast to the well-documented β-AR and ANP receptor desensitization occurring in late-stage cardiac disease such as heart failure.[11,15] Real-time monitoring of cAMP using a novel mouse model expressing the targeted pmEpac1 FRET biosensor demonstrates functional redistribution of PDE2 and 3 between the β_1-AR and β_2-AR-associated membrane microdomains. This redistribution leads to switching of cGMP/cAMP cross-talk from the ANP/cGMP/PDE3-mediated cAMP increase to an ANP/cGMP/PDE2-mediated cAMP decrease after β_2-AR stimulation, and exactly the opposite effect after β_1-AR stimulation (see Figure 4F). Functionally, not only does this lead to a change in physiologically less relevant β_2-AR-specific contractile effects, but strikingly also to stimulatory ANP-effects on global contractile force after β-AR stimulation in hypertrophied myocytes. This effect is apparently caused by a shift in β_1/β_2-AR signal balance towards β_1-AR signaling. Mechanistically, ANP-mediated potentiation of β-AR inotropic response at this stage of disease originates from the reorganization of microdomain-specific PDE activity patterns that cause a shift of ANP-driven cAMP augmenting effect from local (β_2-AR) towards global (β_1-AR) cAMP pools. Unlike pronounced β_1-AR/cAMP and ANP/cGMP signal desensitization in advanced heart failure, our model of early cardiac hypertrophy provides insights into molecular changes which take place during the onset of chronic cardiac disease. Although these changes appear rather neutral with regard to the whole-cell PDE activities, yet we show that even slight changes in local PDE contributions can cause drastic changes in compartmentalized signaling patterns that directly translate into vastly different contractile effects of the same ligand such as ANP (see Figure 1B/C, and Figure 4F). Importantly, the disease-relevant GC-A ligands natriuretic peptides ANP and BNP are present at higher levels in systemic circulation during hypertrophy,

and thus can directly activate cGMP production by not yet desensitized GC-A, thereby generating higher cardiomyocyte cGMP levels and affecting cGMP/cAMP cross-talk to modulate contractility.

Pathophysiologically, this effect might represent a compensatory mechanism to meet the enhanced demand on cardiac output under pressure overload. Strikingly, during early disease, the β_2-AR, which generally plays a minor role in catecholamine-induced inotropic response, generates an important signaling microdomain, which can be regulated by either PDE2 or PDE3 to exert opposite effects on global β-AR-mediated contractility by the altered functional balance between the β_1- and β_2-AR signaling compartments. In this case, an additive effect of both β-AR subtypes on contractile force at healthy state may transform into a relation of reciprocal interdependence (contractile response~β_1-AR-cAMP/β_2-AR-cAMP) in hypertrophy.

In summary, we uncovered a new adaptation mechanism in early cardiac disease which involves subcellular redistribution of cGMP-regulated PDEs. We propose that such alterations well precede well-known cellular remodelling which occurs during decompensated hypertrophy and heart failure associated with changed whole-cell PDE activities and the desensitization of the β_1-AR/cAMP and ANP/cGMP signaling cascades. This mechanism might become a new target for therapeutics acting on natriuretic peptides and microdomain-specific signaling in early disease.

ACKNOWLEDGMENTS
We thank the transgenic core unit of the Max-Planck-Institute for Experimental Medicine for transgenic mouse generation, Christian Dees for radioligand binding assays, Karina Zimmermann for support in histology and immunoblot analysis, Kirsten Koschel and Sabrina Wollborn for echocardiography, Mei-Ling Chang Liao for advice on IonOptix, and Jan-Hendrik Streich for advice on heart rate measurements.

SOURCES OF FUNDING
The work was supported by the Deutsche Forschungsgemeinschaft (grants FOR2060 and NI 1301/1 to V.O.N., SFB 1002 to A.E.A., S.E.L. and V.O.N.; and KFO 155 to S.E.L.) and the University of Göttingen Medical Center ("pro futura" grant to V.O.N.).

DISCLOSURES
None.

REFERENCES

1. Perino A, Ghigo A, Scott JD, Hirsch E. Anchoring proteins as regulators of signaling pathways. *Circ Res*. 2012;111:482-492
2. Perera RK, Nikolaev VO. Compartmentation of cAMP signalling in cardiomyocytes in health and disease. *Acta Physiol (Oxf)*. 2013;207:650-662
3. Zaccolo M, Movsesian MA. cAMP and cGMP signaling cross-talk: role of phosphodiesterases and implications for cardiac pathophysiology. *Circ Res*. 2007;100:1569-1578
4. Stangherlin A, Gesellchen F, Zoccarato A, Terrin A, Fields LA, Berrera M, Surdo NC, Craig MA, Smith G, Hamilton G, Zaccolo M. cGMP signals modulate cAMP levels in a compartment-specific manner to regulate catecholamine-dependent signaling in cardiac myocytes. *Circ Res*. 2011;108:929-939
5. Xiao RP. Beta-adrenergic signaling in the heart: dual coupling of the beta2-adrenergic receptor to G(s) and G(i) proteins. *Sci STKE*. 2001;2001:re15
6. Nikolaev VO, Moshkov A, Lyon AR, Miragoli M, Novak P, Paur H, Lohse MJ, Korchev YE, Harding SE, Gorelik J. Beta2-adrenergic receptor redistribution in heart failure changes cAMP compartmentation. *Science*. 2010;327:1653-1657
7. Tsai EJ, Kass DA. Cyclic GMP signaling in cardiovascular pathophysiology and therapeutics. *Pharmacol Ther*. 2009;122:216-238
8. Holtwick R, van Eickels M, Skryabin BV, Baba HA, Bubikat A, Begrow F, Schneider MD, Garbers DL, Kuhn M. Pressure-independent cardiac hypertrophy in mice with cardiomyocyte-restricted inactivation of the atrial natriuretic peptide receptor guanylyl cyclase-A. *J Clin Invest*. 2003;111:1399-1407
9. Knowles JW, Esposito G, Mao L, Hagaman JR, Fox JE, Smithies O, Rockman HA, Maeda N. Pressure-independent enhancement of cardiac hypertrophy in natriuretic peptide receptor A-deficient mice. *J Clin Invest*. 2001;107:975-984
10. Bristow MR, Ginsburg R, Minobe W, Cubicciotti RS, Sageman WS, Lurie K, Billingham ME, Harrison DC, Stinson EB. Decreased catecholamine sensitivity and beta-adrenergic-receptor density in failing human hearts. *N Engl J Med*. 1982;307:205-211
11. Kuhn M. Structure, regulation, and function of mammalian membrane guanylyl cyclase receptors, with a focus on guanylyl cyclase-A. *Circ Res*. 2003;93:700-709
12. Börner S, Schwede F, Schlipp A, Berisha F, Calebiro D, Lohse MJ, Nikolaev VO. FRET measurements of intracellular cAMP concentrations and cAMP analog permeability in intact cells. *Nat Protoc*. 2011;6:427-438
13. Gotz KR, Sprenger JU, Perera RK, Steinbrecher JH, Lehnart SE, Kuhn M, Gorelik J, Balligand JL, Nikolaev VO. Transgenic Mice for Real-Time Visualization of cGMP in Intact Adult Cardiomyocytes. *Circ Res*. 2014;114:1235-1245
14. Takimoto E, Belardi D, Tocchetti CG, Vahebi S, Cormaci G, Ketner EA, Moens AL, Champion HC, Kass DA. Compartmentalization of cardiac beta-adrenergic inotropy modulation by phosphodiesterase type 5. *Circulation*. 2007;115:2159-2167
15. Lohse MJ, Engelhardt S, Eschenhagen T. What is the role of beta-adrenergic signaling in heart failure? *Circ Res*. 2003;93:896-906
16. Lainchbury JG, Burnett JC, Jr., Meyer D, Redfield MM. Effects of natriuretic peptides on load and myocardial function in normal and heart failure dogs. *Am J Physiol Heart Circ Physiol*. 2000;278:H33-40
17. Ohte N, Cheng CP, Suzuki M, Little WC. Effects of atrial natriuretic peptide on left ventricular performance in conscious dogs before and after pacing-induced heart failure. *J Pharmacol Exp Ther*. 1999;291:589-595
18. Abi-Gerges A, Richter W, Lefebvre F, Mateo P, Varin A, Heymes C, Samuel JL, Lugnier C, Conti M, Fischmeister R, Vandecasteele G. Decreased expression and activity of cAMP

phosphodiesterases in cardiac hypertrophy and its impact on beta-adrenergic cAMP signals. *Circ Res*. 2009;105:784-792
19. Ding B, Abe J, Wei H, Huang Q, Walsh RA, Molina CA, Zhao A, Sadoshima J, Blaxall BC, Berk BC, Yan C. Functional role of phosphodiesterase 3 in cardiomyocyte apoptosis: implication in heart failure. *Circulation*. 2005;111:2469-2476
20. Castro LR, Verde I, Cooper DM, Fischmeister R. Cyclic guanosine monophosphate compartmentation in rat cardiac myocytes. *Circulation*. 2006;113:2221-2228
21. Mehel H, Emons J, Vettel C, Wittkopper K, Seppelt D, Dewenter M, Lutz S, Sossalla S, Maier LS, Lechene P, Leroy J, Lefebvre F, Varin A, Eschenhagen T, Nattel S, Dobrev D, Zimmermann WH, Nikolaev VO, Vandecasteele G, Fischmeister R, El-Armouche A. Phosphodiesterase-2 Is up-regulated in human failing hearts and blunts beta-adrenergic responses in cardiomyocytes. *J Am Coll Cardiol*. 2013;62:1596-1606
22. Richter W, Day P, Agrawal R, Bruss MD, Granier S, Wang YL, Rasmussen SG, Horner K, Wang P, Lei T, Patterson AJ, Kobilka B, Conti M. Signaling from beta1- and beta2-adrenergic receptors is defined by differential interactions with PDE4. *Embo J*. 2008;27:384-393
23. Patrucco E, Albergine MS, Santana LF, Beavo JA. Phosphodiesterase 8A (PDE8A) regulates excitation-contraction coupling in ventricular myocytes. *J Mol Cell Cardiol*. 2010;49:330-333
24. Shakur Y, Takeda K, Kenan Y, Yu ZX, Rena G, Brandt D, Houslay MD, Degerman E, Ferrans VJ, Manganiello VC. Membrane localization of cyclic nucleotide phosphodiesterase 3 (PDE3). Two N-terminal domains are required for the efficient targeting to, and association of, PDE3 with endoplasmic reticulum. *J Biol Chem*. 2000;275:38749-38761

FIGURE LEGENDS

Figure 1. Contractile responses to ANP in healthy and hypertrophied cardiomyocytes. Cells were isolated from mice 8 weeks after transverse aortic constriction (TAC) or sham operation (Sham) and electrically stimulated at 1 Hz. Sarcomere shortening was monitored under application of low dose of the β-AR agonist isoproterenol (ISO, 3 nmol/L) and 100 nmol/L of atrial natriuretic peptide (ANP) to sham (*A*) and TAC (*B*) cells. ANP does not significantly affect (shows only a tendency for a slight decrease) contractility in healthy but significantly increases it in hypertrophied cells. The unselective PDE blocker 3-Isobutyl-1-methylxanthine (IBMX, 100 µmol/L) was used as a positive control. *C*, Quantification of experiments shown in A and B as a % of basal contractility at peak response. In addition, the responses to 100 nmol/L ANP alone are presented. n=3 Sham and 3 TAC mice. *D*, ANP applied after ISO increases heart rates in intact Langendorff-perfused TAC but not sham hearts. *E*, In vivo hemodynamic measurements reveal a strong tendency of increased cardiac output and significantly increased heart rates (*F*) in TAC mice infused with ANP and ISO administration. Means ± SE, number of cells or hearts and p-values are indicated above the bars. Functional parameters of sham and TAC mice are presented in Online Figure V.

Figure 2. ANP augments ISO-induced cAMP signals in hypertrophied cardiomyocytes. *A*, Cells were isolated from pmEpac1 mice 8 weeks after TAC or sham surgery. ANP effect on cAMP levels after prestimulation with 3 nmol/L of ISO was greatly enhanced in TAC myocytes. Quantification of ANP effect after ISO, along with the data for ISO stimulation alone is in *B*, means ± SE, number of cells and p-values are indicated above the bars. *C*, FRET recordings in TAC and sham cells during stimulation with the β-AR agonist ISO (100 nmol/L), followed by the maximal stimulation with the direct adenylyl cyclase activator forskolin (10 µmol/L) plus IBMX (100 µmol/L). (*D*, *E*) β_1- and β_2-ARs were selectively stimulated with 100 nmol/L of ISO plus 50 nmol/L of the β_2-AR antagonist ICI118551 (ICI) and with ISO plus 100 nmol/L of the β_1-AR antagonist CGP20712A (CGP), respectively. *F*, Quantification of the FRET results showed increased β_1- and decreased β_2-AR-cAMP responses after TAC, and no differences between ISO and β_1-AR selective stimulations, means ± SE, number of cells and p-values are indicated above the bars.

Figure 3. PDE2 and PDE3 redistribute between β_1- and β_2-AR-associated cAMP microdomains in cardiac hypertrophy. (*A*, *B*) Representative traces showing cilostamide (Cilo, 10 µmol/L) and BAY60-7550 (BAY, 100 nmol/L) effects in TAC and sham pmEpac1 cardiomyocytes after global ISO stimulation. *C*, Quantifications of the PDE inhibitor signals (as a % of the maximal response evoked by the unselective inhibitor IBMX) obtained after ISO stimulation in TAC and sham cells. *D*, After selective β_2-AR (ISO+CGP) prestimulation, PDE3 inhibitor effects are dramatically reduced while PDE2 inhibitor responses are markedly increased in hypertrophied cells (*E*). *F*, Quantifications of the PDE inhibitor signals after β_2-AR selective stimulation. Shown are means ± SE, number of cells and p-values for significant differences are indicated above the bars.

Figure 4. Altered cGMP dependent regulation of membrane β_2-AR- and β_1-AR-cAMP levels in cardiac hypertrophy. *A*, Addition of atrial natriuretic peptide (ANP, 100 nmol/L) after selective β_2-AR stimulation (ISO+CGP) leads to further significant increase in cAMP. Pretreatment of cells with 10 µmol/L of the selective PDE3 inhibitor cilostamide (Cilo) prevents the effect of ANP, suggesting a PDE3-dependent mechanism. *B*, Quantification of the ANP responses shown in A. Means ± SE, number of cells and p-values for significant differences are indicated above the bars. *C*, The effect of ANP is abolished in diseased myocytes but can be restored after preincubation with the PDE2 inhibitor BAY60-7550 (BAY, 100 nmol/L). *D*, Quantification of the ANP effect in TAC and sham cells as a % of maximal PDE3 inhibition with cilostamide. *E*, Changes in membrane cAMP directly translate into contractile response. Analysis of the sarcomere shortening data shown in Online Figure XIV and of the cGMP-AM effect (100 µmol/L) under the same conditions, means ± SE, number of cells and p-values for significant differences are indicated above the bars. *F*, Schematic representation of the proposed molecular switch mechanism.

Redistribution of PDE2 from the β_1- to the β_2-AR-associated membrane microdomains leads to a decrease of the local β_2-AR-cAMP and to an increase of global β_1-AR-cAMP pool under elevated ANP and cGMP levels observed in early disease. The switch of β_2-AR-associated PDE2 and 3 leads to a turnaround of cGMP/cAMP cross-talk between the β_2- and β_1-AR-associated microdomains, through which elevated ANP augments β-adrenoceptor-stimulated contractility under shifted β_1-/β_2-AR-cAMP balance. NE, norepinephire, the physiological β-AR agonist.

DOI: 10.1161/CIRCRESAHA.116.306082

Novelty and Significance

What Is Known?

- Cyclic nucleotides 3',5'-cylcic adenosine (cAMP) and guanosine (cGMP) monophosphates play crucial role in regulating cardiac contractile function by acting in discrete subcellular microdomains.

- Chronic decompensated cardiac disease such as heart failure is associated with attenuated cAMP and cGMP signaling from β-adrenergic (β-AR) and natriuretic peptide receptors, respectively.

- The role of cyclic nucleotide microdomains in cardiac disease is poorly understood, especially not at an early functionally compensated stage, which is most relevant for therapeutic interventions

What New Information Does This Article Contribute?

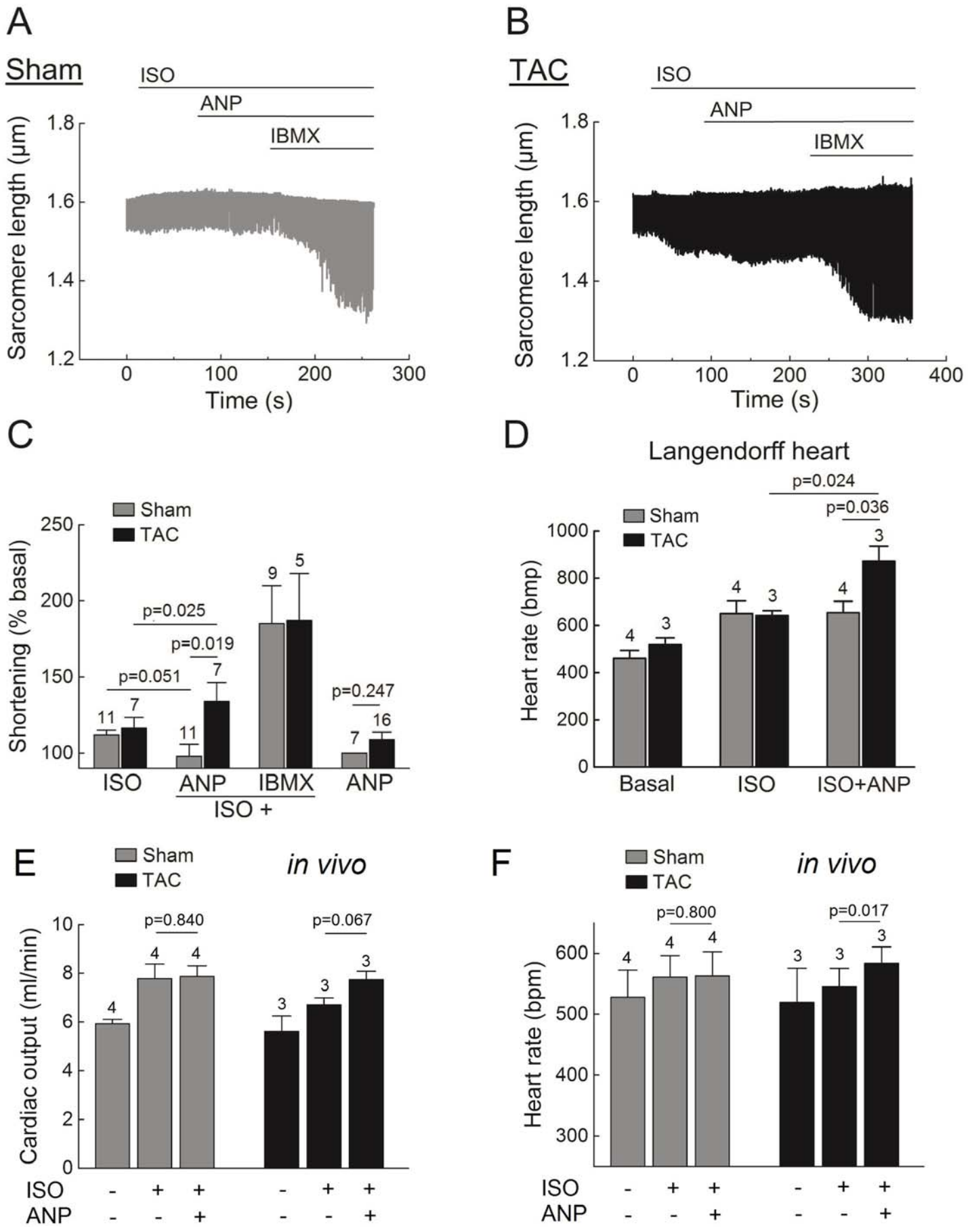
- A novel transgenic mouse which expresses a highly sensitive Förster resonance energy transfer (FRET)-based cAMP biosensor targeted to caveolin-rich membrane microdomains.

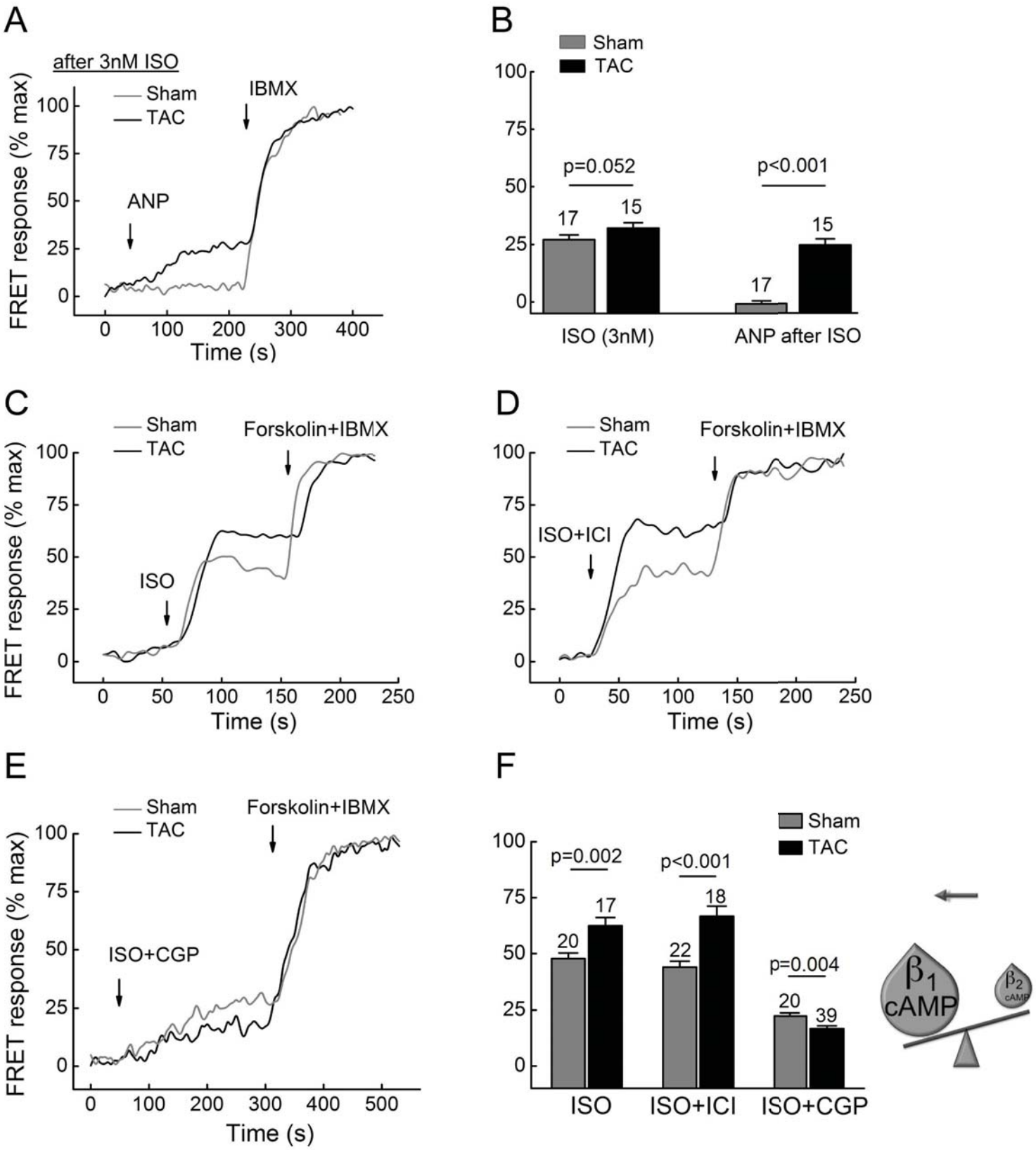
- In contrast to chronic disease, early cardiac hypertrophy is associated with an augmenting effect of the atrial natriuretic peptide (ANP) on β_1-AR induced force (in vitro) and frequency (in vitro and in vivo) of contraction due to a redistribution of cGMP-regulated phosphodiesterases 2 and 3 between the β_1- and β_2-AR-associated membrane microdomains.

- This remarkable effect of ANP is brought about exclusively by subcellular, microdomain-specific alterations of phosphodiesterase localization and activity, while their whole-cell expression levels and β-AR densities are not yet altered at this stage of disease

In this work, we sought to study the role of cyclic nucleotide microdomains in early cardiac disease. We generated novel transgenic mice expressing a highly-sensitive membrane-targeted FRET biosensor for cAMP and subjected these animals to transverse aortic constriction to induce compensated cardiac hypertrophy. In sharp contrast to well-documented β-AR and ANP receptor desensitization occurring in chronic disease, we uncovered a positive inotropic effect of ANP in isolated myocytes and its positive chronotropic effect in isolated hearts and in vivo, which augmented β_1-AR effects due to a redistribution of therapeutically relevant cGMP-regulated phosphodiesterases between the β_1- and β_2-AR-associated membrane microdomains. These subcellular alterations were not yet accompanied by whole-cell changes in phosphodiesterase activities and β-AR densities but already affected contractility, indicating a new clinically interesting compensatory mechanism which might transiently support contractile function during pressure overload before the transition to a decompensated chronic disease. Our data may have direct translational implications for the stage-dependent heart failure therapy using new neprilysin inhibitor drugs which act by increasing circulating ANP levels.

DOI: 10.1161/CIRCRESAHA.116.306082





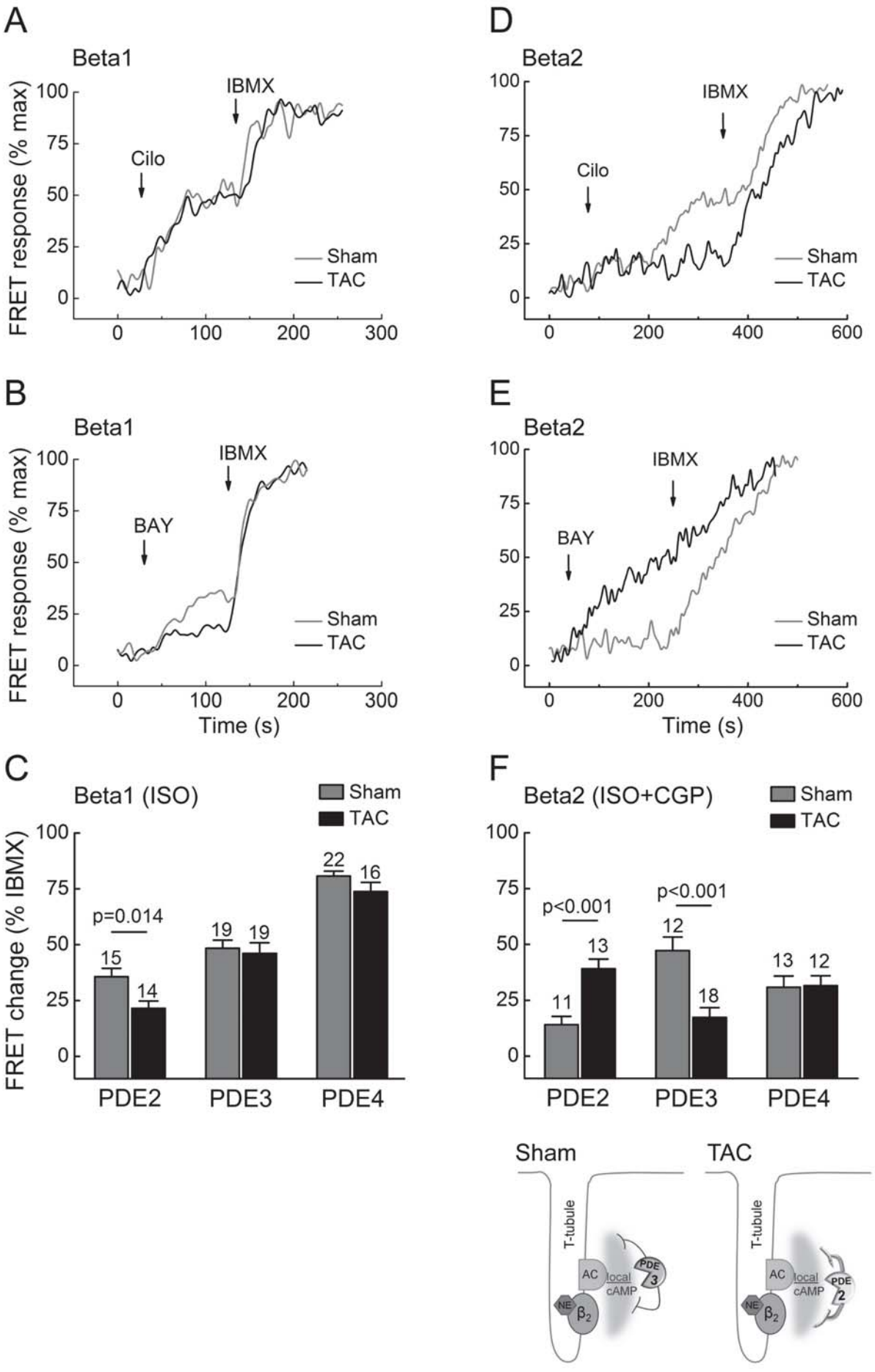
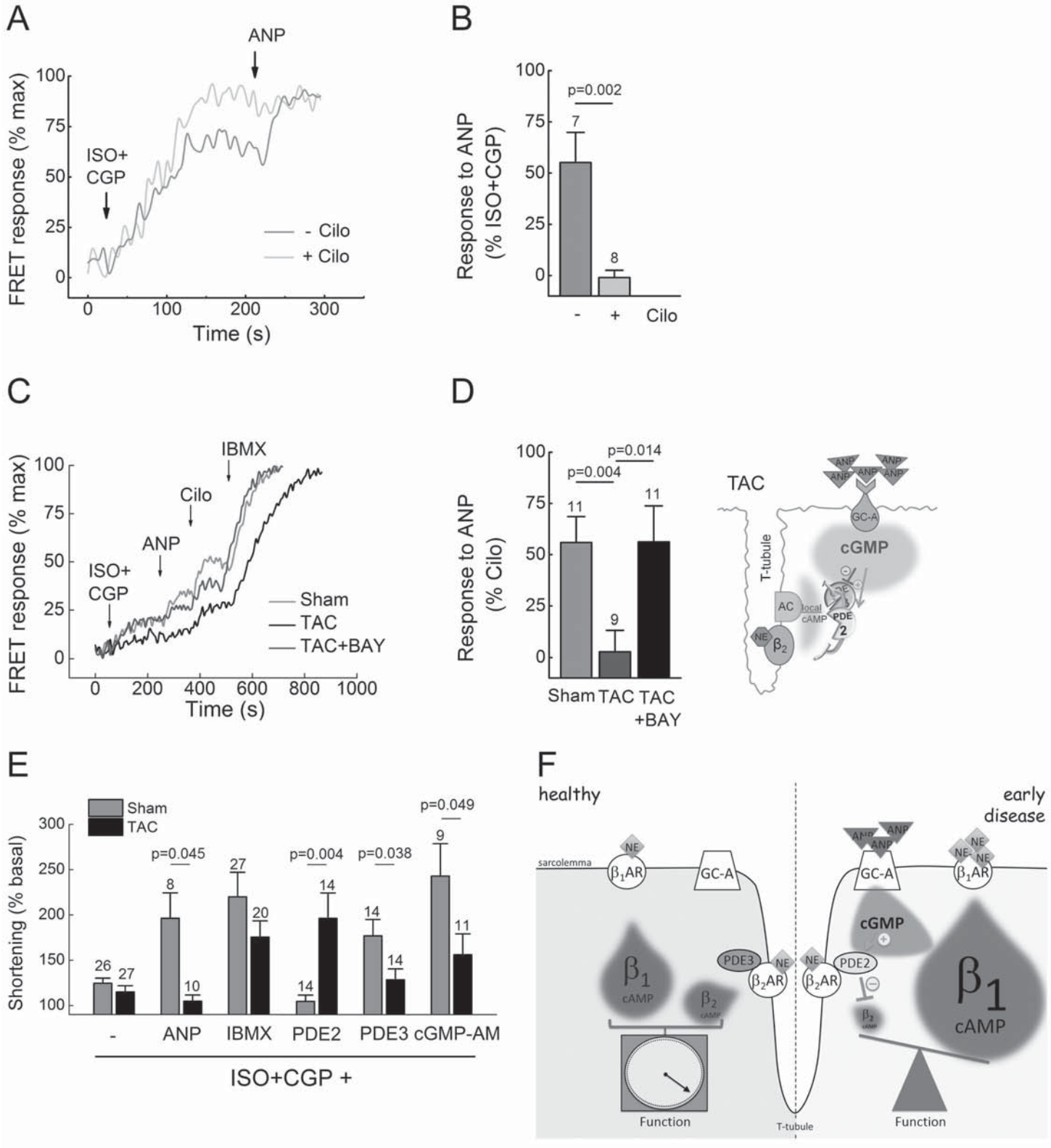


Figure 4



SUPPLEMENTAL MATERIAL TO MANUSCRIPT: CIRCRES/2015/306082/R1

Brief UltraRapid Communication

Microdomain switch of cGMP-regulated phosphodiesterases leads to ANP-induced augmentation of β-adrenoceptor-stimulated contractility in early cardiac hypertrophy

Perera. Molecular basis of hypercontractility

Ruwan K. Perera,[1,2,3], PhD, Julia U. Sprenger,[1,2,3], PhD, Julia H. Steinbrecher,[2] Ms, Daniela Hübscher,[1,2], PhD, Stephan E. Lehnart,[2,4] MD, Marco Abesser,[5] Mr, Kai Schuh,[5] PhD, Ali El-Armouche,[2,6] MD, and Viacheslav O. Nikolaev,[1,2,3,4*] PhD

[1]Emmy Noether Group of the DFG, European Heart Research Institute Göttingen; [2]Heart Research Center Göttingen, Georg August University Medical Center, D-37075 Göttingen, Germany; [3]Institute of Experimental Cardiovascular Research, University Medical Center Hamburg-Eppendorf, D-20246 Hamburg, Germany; [4]German Center for Cardiovascular Research (DZHK); [5]Instutite of Physiology, University of Würzburg, D-97070 Würzburg, Germany; [6]Institute of Pharmacology and Toxicology, Dresden University of Technology, D-01307 Dresden, Germany

*Address for correspondence:

Prof. Dr. Viacheslav O. Nikolaev
Institute of Experimental Cardiovascular Research
University Medical Center Hamburg-Eppendorf
Martinistr. 52
D-20246 Hamburg, Germany
Phone: +49-40-7410-51391; Fax: +49-40-7410-40180
E-mail: v.nikolaev@uke.de

Detailed Methods

Cloning and transgenic mouse generation. DNA encoding the N-terminal 10 amino acid peptide MGSINSKRKD from the Lyn kinase[1] was subcloned upstream of the start ATG of the Epac1-camps sensor[2] using the HindIII and KpnI restriction sites (the latter resulting in the addition of the additional two amino acids AS), see Online Figure 2A for details. The whole sensor sequence was transferred into the previously described vector containing the αMHC promoter and simian virus (SV40) polyadenylation signal.[3] The resulting vector was linearized with SpeI, purified and used for pronuclear injections to generate transgenic mice on the FVB/N background as described.[4] The resultant founder mice and their heterozygote offspring were genotyped by a standard PCR using the primers TGACAGACAGATCCCTCCTAT and CATGGCGGACTTGAAGAAGT, resulting in a ~365 b.p. fragment on a gel.

Histology, morphometric analysis and echocardiography were performed as previously described.[5] For cardiomyocyte dimension analysis, transverse heart sections were incubated with Wheat Germ Agglutinin (WGA, 75 µg/mL) for 30 min in the dark, washed thrice for 5 min with phosphate buffered saline, mounted and observed under Axiovert 200 microscope (Carl Zeiss MicroImaging, Jena, Germany). Images were acquired using AxioVision software (Carl Zeiss MicroImaging) and analyzed with ImageJ software. The cell diameter was measured in 100 cells from 5 sections per heart.

Transverse aortic constriction (TAC). 9-13 week aged female mice were randomized into sham or TAC group. Mice were anesthetized using 1.5-2 % isoflurane in 100 % oxygen. A suprasternal incision was made, and the aortic arch was visualized using a binocular macroscope (Olympus). TAC occurred by spacer defined (26-gauge) constriction using a 6-0 polyviolene suture between the first and second trunk of the aortic arch.[6] For sham, the aorta was exposed but not constricted. 3 days after surgery, Doppler velocity was measured by a 20 MHz probe to quantify the pressure gradient across the TAC region or after sham procedure by transthoracic echocardiography (VisualSonics Vevo 2100; Toronto, Canada). 3 days before and during 1 week after surgery, mice received analgesic therapy with metamizole (1.33 mg/ml in drinking water). Echocardiography was performed 8 weeks after surgery with subsequent heart and cell isolation. The data presented in Online Figure V demonstrate a mild functionally compensated phenotype. The mildness of the phenotype can be explained by the FVB/N1 stain of mice used which is less susceptible to heart failure development after TAC than other mouse strains.

Single-cell contractility measurements. Freshly isolated cardiomyocytes were plated onto laminin-coated glass coverslides. Contractile responses were evaluated by sarcomere length measurements method (IonOptix) at 1 Hz pacing frequency as previously described.[7]

***In vivo* hemodynamic measurements**. Recordings of left ventricular pressure volume loops were performed in 1% isoflurane anesthetized mice with a 1.4 F catheter connected to an MPVS Ultra amplifier (Millar Instruments), as previously described[8]. After 10 min of stabilization, mice were infused for 10 min with lactated Ringer's solution at a rate of 0.26 µl/g/min, subsequently 10 min with Isoproterenol at 10 pg/g/min, and finally with a mixture of Isoproterenol (10 pg/g/min) and ANP (500 pg/g/min) in Ringer's solution through the jugular vein. Data were collected and analyzed with a PowerLab 16/30 and Chart 7.3 (ADInstruments).

Confocal microscopy was performed using Zeiss LSM 710 NLO microscope (Carl Zeiss MicroImaging) equipped with a Plan-Apochromat x63/1.40 oil-immersion objective. For live cell imaging, myocytes were incubated with 50 µmol/L of di-8-ANEPPS for 10-15 min. Images were acquired for CFP/YFP (405 nm diode laser excitation) and di-8-ANEPPS (488 nm argon ion laser excitation) and analyzed using ZEN 2010 software (Zeiss). For co-localization experiments, cells were fixed for 20 min with ice-cold ethanol at -20°C, washed and co-stained with mouse monoclonal α-actinin antibody (Sigma) and either goat polyclonal anti-PDE2A (sc-

17228, Santa Cruz) or goat polyclonal anti-PDE3A (sc-11834, Santa Cruz) antibodies, followed by the secondary anti-mouse Alexa 633 Fluor® and anti-goat Alexa 488 Fluor® antibodies (A-21063 and A-11055, respectively, Life Technologies). Images were taken and automatically analyzed using the ZEN 2010 software to calculate the Pearson's coefficient which shows the degree of co-localization. For staining of neonatal rat cardiomyocytes, cells were isolated as previously described[9] and transduced with adenoviruses expressing HA-tagged β_1-AR or FLAG-tagged β_2-AR (kind gift from Dr. Y. K. Xiang) and cultured for 48 h in presence or absence 50 µM phenylephrine (PE) to induce hypertrophy. Thereafter, cells were fixed with methanol/acetone and co-stained with PDE2A/3A and rabbit anti-HA (Covance) or mouse anti-FLAG (Sigma) antibodies for confocal microscopy.

Radioligand binding. Radioligand binding studies were performed as previously described.[10] Briefly, isolated cell membranes were incubated for 1 h at 30°C with 60-100 pmol/L ^{125}I-cyanopindolol (PerkinElmer Life Sciences, Dreieich, Germany) and increasing concentrations of ICI118551.

Phosphodiesterase activity assay. Freshly isolated cardiomyocytes were lysed and processed for *in vitro* measurement of cAMP-PDE hydrolyzing activity following the standard method by Thompson and Appleman in presence of 1 µmol/L cAMP as a substrate, as previously described.[11,12] Contributions of individual PDE families were calculated from the effects of 100 nmol/L BAY (PDE2), 10 µmol/L cilostamide (PDE3), 10 µmol/L rolipram (PDE4), and 100 µmol/L IBMX (unselective inhibitor).

Immunoblot analysis. Freshly isolated cardiomyocytes or heart tissues were shock frozen and homogenized in a buffer containing in mmol/L: 10 HEPES, 300 succrose, 150 NaCl, 1 EGTA, 2 $CaCl_2$, and 1 % Triton-X. Proteins were quantified using BCA Protein Assay (Pierce). Samples were boiled at 95°C for 5 minutes, and 10 µg of total protein per lane were subjected to 10 % or 4-12 % (Bis-AA gradient gels, Criterion) SDS-PAGE and to immunoblot analysis using anti-PDE2 antibody (Fabgennix), rabbit polyclonal PDE4D8 antibody (kind gift from Marco Conti), rabbit polyclonal PDE8A antibody (kind gift from George Baillie), goat polyclonal β-arrestin 2 antibody (Abcam), rabbit polyclonal PDE3A antibody (kind gift from Chen Yan), rabbit polyclonal AKAP13 antibody (kind gift from Michael Kapiloff), mouse polyclonal PI3Kγ antibody (kind gift from Emilio Hirsch), mouse monoclonal caveolin 3 antibody (Sigma), rabbit polyclonal calsequestrin (Thermo Scientific) and a monoclonal GAPDH antibody (HyTest). To analyze PKA substrate phopshorylation, myocytes isolated from TAC and sham mice were stimulated for 5 min with vehicle, 100 nmol/L ISO or 100 nmol/L ISO plus 100 nmol/L ANP, lysed and subjected to immunoblot analysis using phospho-phospholamban (Ser16, Badrilla) and phospho-myosin binding protein C (Enzo) antibodies. All blots were scanned and analyzed densitometrically by ImageJ software for uncalibrated optical density.

Subcellular fractionation was performed according to the previously described method with minor modifications[13]. Sham and TAC heart lysates were mixed with MES-buffered saline (25 mmol/L MES and 150 mmol/L NaCl, pH=6.5) containing 250 mmol/L Na_2CO_3 and applied onto sucrose gradient. 10 µg protein from each fraction were loaded onto 4-12 % gradient gel (Bis-AA Criterion) and further processed for immunoblot analysis with the antibodies described above plus β_1-AR (sc-568) and β_2-AR (sc-569) antibodies from Santa Cruz.

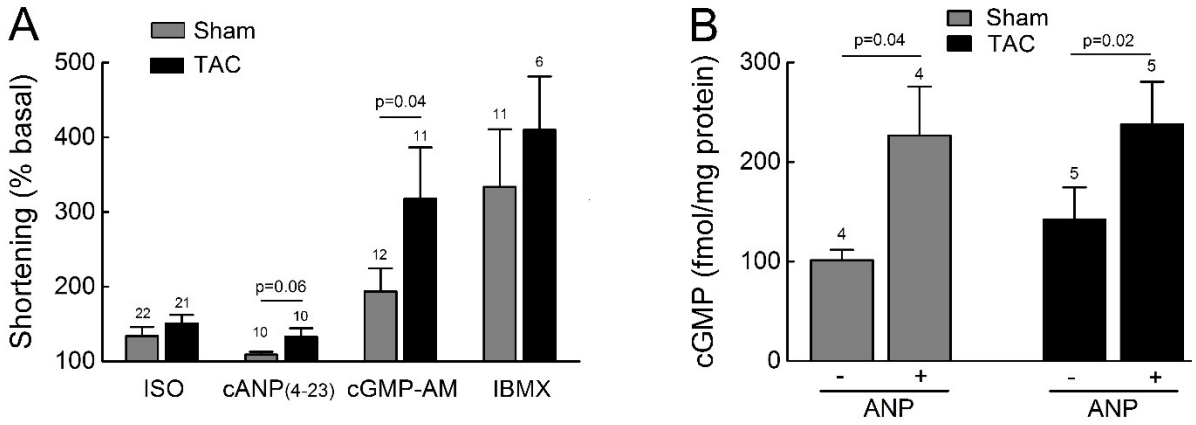
cGMP radioimmunoassay. Freshly isolated adult cardiomyocytes from sham and TAC mice were resuspended in IBMX-containing (500 µmol/L) Tyrode's solution. After 15 min the samples were stimulated for 10 min with 100 nmol/L ANP or vehicle, lysed with ethanol and processed for radioimmunoassay analysis as previously described.[14,15]

Contraction rate measurements in intact hearts. TAC and sham mice were sacrificed by cervical dislocation. Hearts were rapidly explanted and subjected to Langendorff perfusion with the Krebs-Henseleit solution (in mmol/L: 118 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.25 $MgSO_4$, 24

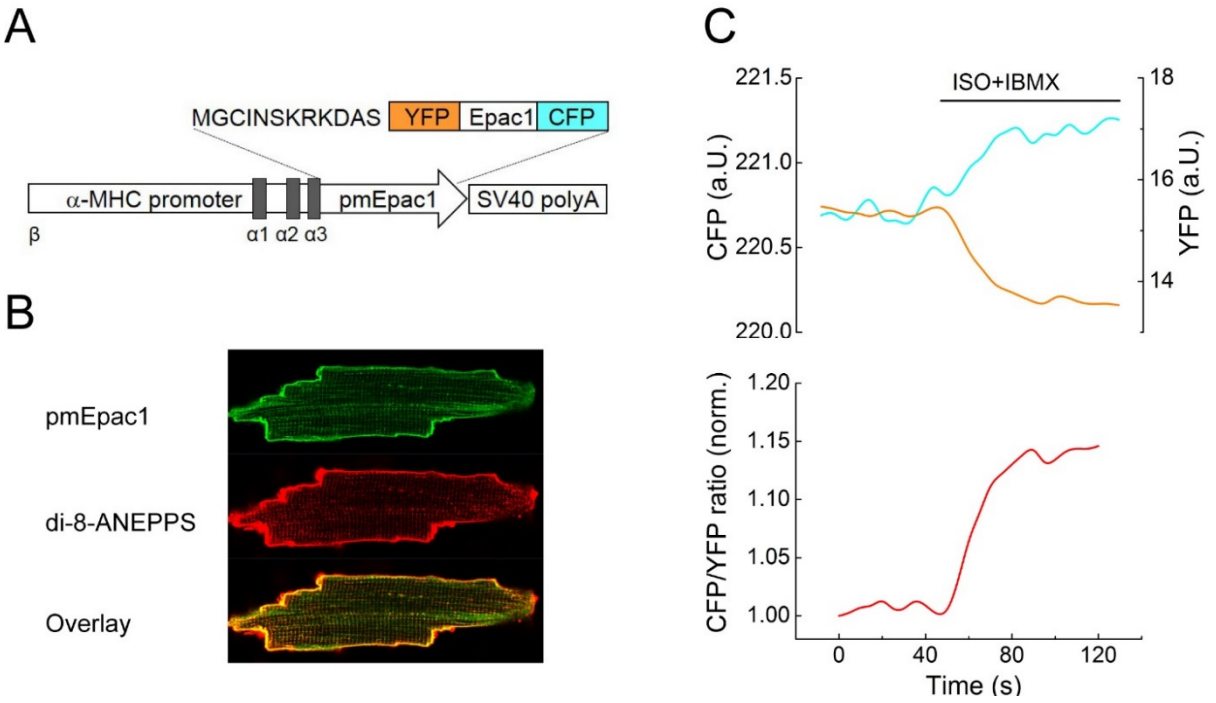
$NaHCO_3$, 1.25 $CaCl_2$ and 11.1 glucose; constantly oxygenated with 95 % O_2 and 5 % CO_2) at 37.5 °C with a constant flow rate of 2.5 ml/min. Heart beats were detected with a custom made electrode and analyzed with Powerlab Chart 5 software (ADInstruments). Hearts were perfused for 3-5 minutes to reach stable baseline and then stimulated with 10 nmol/L ISO for 3-5 min. After reaching the plateau phase, 100 nmol/L of ANP were additionally washed in for another 3-5 min.

Myocardial infaction (MI) model involving left anterior descending coronary artery ligation to induce chronic heart failure in mice was performed exactly as previously described.[5] Operated animals were subjected to echocardiography and subsequent cardiomyocyte isolation 12 weeks post-MI.

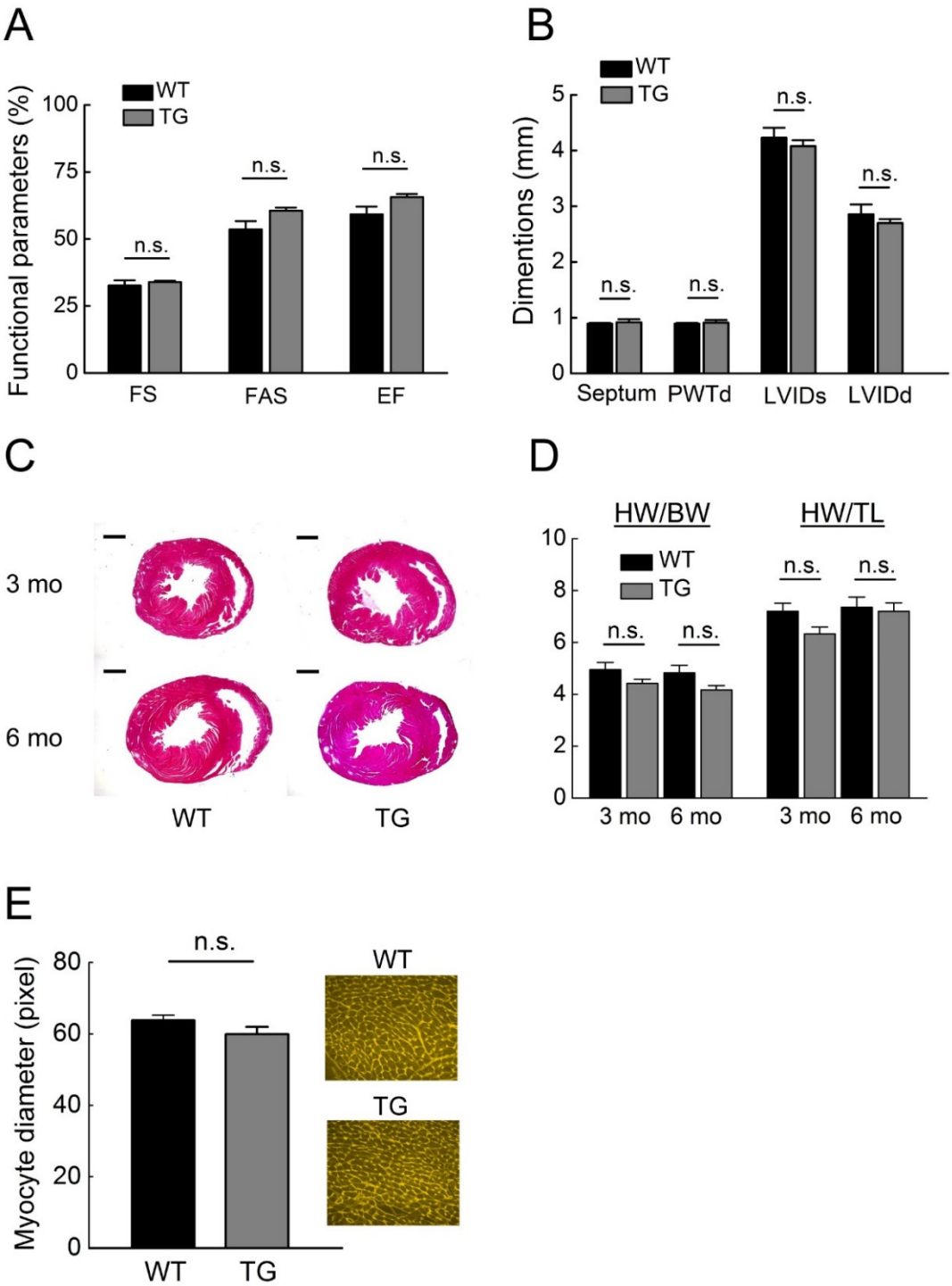
Supplemental Figures



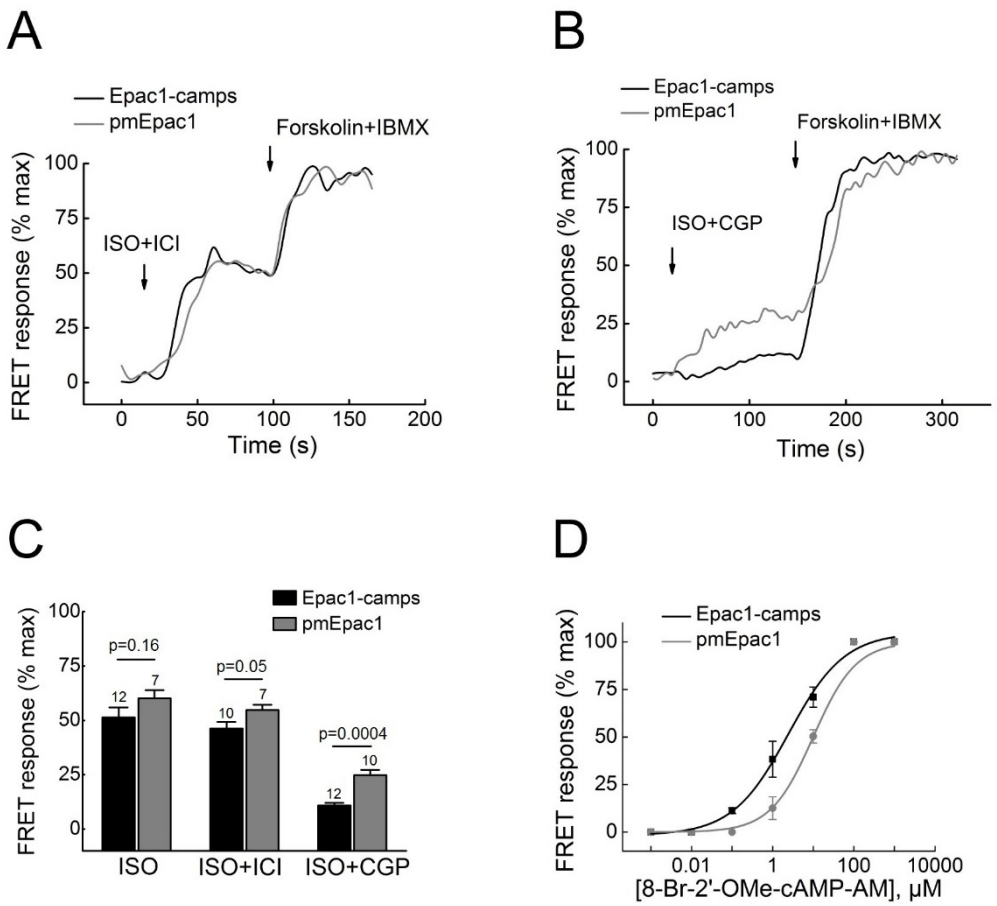
Online Figure I. *Measurements of sarcomere shortening in cells stimulated with the NPR-C receptor agonist and cGMP analogue, as well as cGMP levels in response to ANP.* (*A*) Cells from mice 8 weeks after transverse aortic constriction (TAC) or sham operation (sham) were paced at 1 Hz and stimulated first with a low dose of the β-AR agonist isoproterenol (ISO, 3 nmol/L) and subsequently with 100 nmol/L of the NPR-C receptor agonist cANP(4-23) or 100 µmol/L of the cell-permeable cGMP analogue cGMP-AM. Shown are means ± SE, number of cells and p-values are indicated above the bars. NPR-C is a clearance receptor for natriuretic peptides which lacks guanylyl cyclase domain but has been shown to engage some additional signaling pathways such as inhibitory G-proteins.[16] (*B*) TAC and sham cells were stimulated for 10 min with 100 nmol/L ANP or vehicle in presence of IBMX, and lysed for radioimmunoassay measurements of cGMP content. Averaged data from 4 sham and 5 TAC mice are presented as means ± SE. Differences between sham and TAC groups in basal and stimulated cGMP levels are not significant at p=0.05.



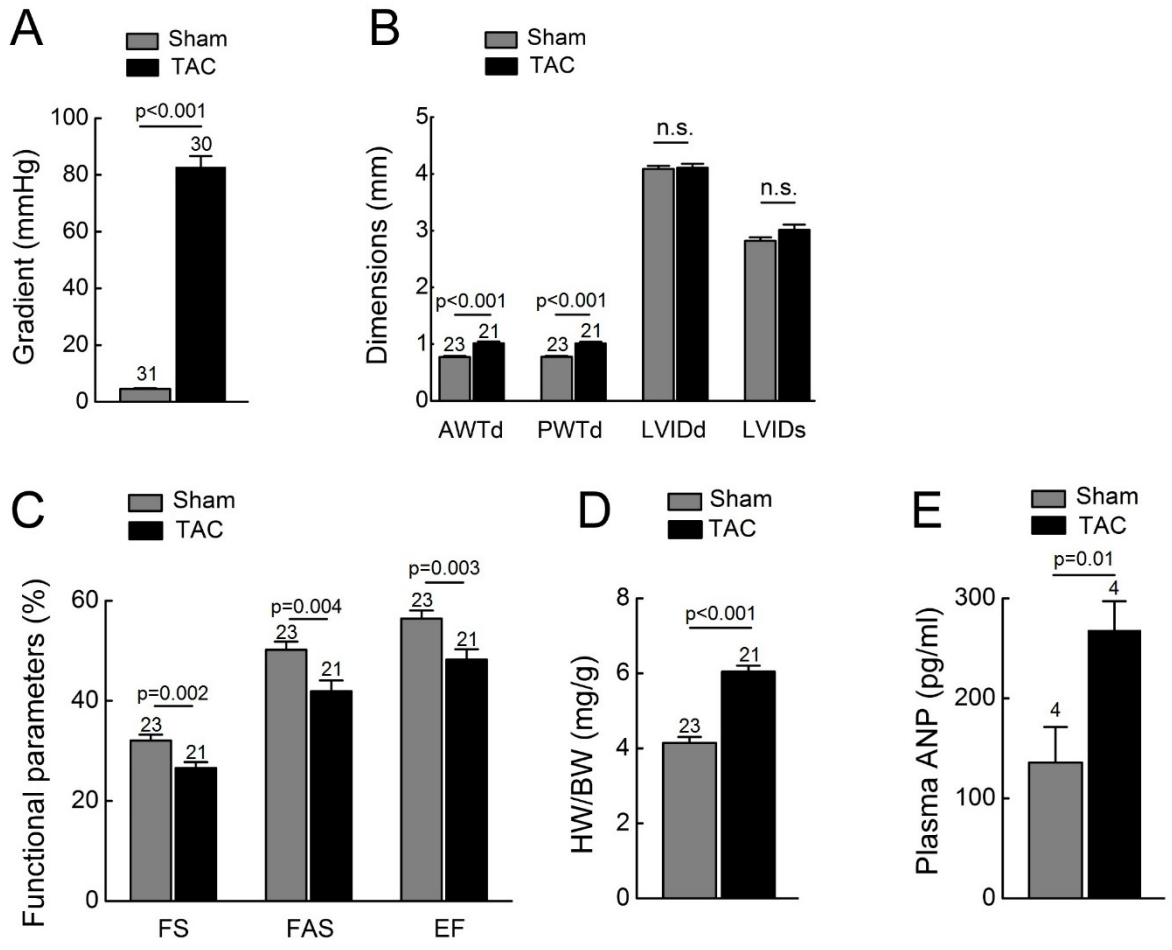
Online Figure II. *Novel pmEpac1 transgenic mouse.* (*A*) Schematic representation of the construct used for pronuclear injections. The sensor sequence is expressed under the control of the α-MHC promoter and followed by the simian virus (SV40) polyadenylation signal (polyA). We used a membrane-targeted version of the highly sensitive FRET-based cAMP sensor Epac1-camps (called pmEpac1), which was anchored to caveolin-rich membrane regions by the well-established N-terminal 10 amino acid sequence from the Lyn kinase encoding palmitoylation and myristoylation motifs.[1] (*B*) Confocal analysis of sensor localization in freshly isolated adult mouse cardiomyocytes by live-staining with the membrane dye di-8-ANEPPS. (*C*) Representative FRET recording from a single cardiomyocyte. Stimulation with the β-adrenergic receptor (β-AR) agonist isoproterenol (ISO, 100 nmol/L) and the unselective phosphodiesterase inhibitor IBMX (100 µmol/L) causes a rapid increase in the donor (CFP) and a decrease of acceptor (YFP) fluorescence, indicative of a decrease in FRET, whereby the CFP/YFP ratio reflects the real time cAMP dynamics in the membrane microdomain.



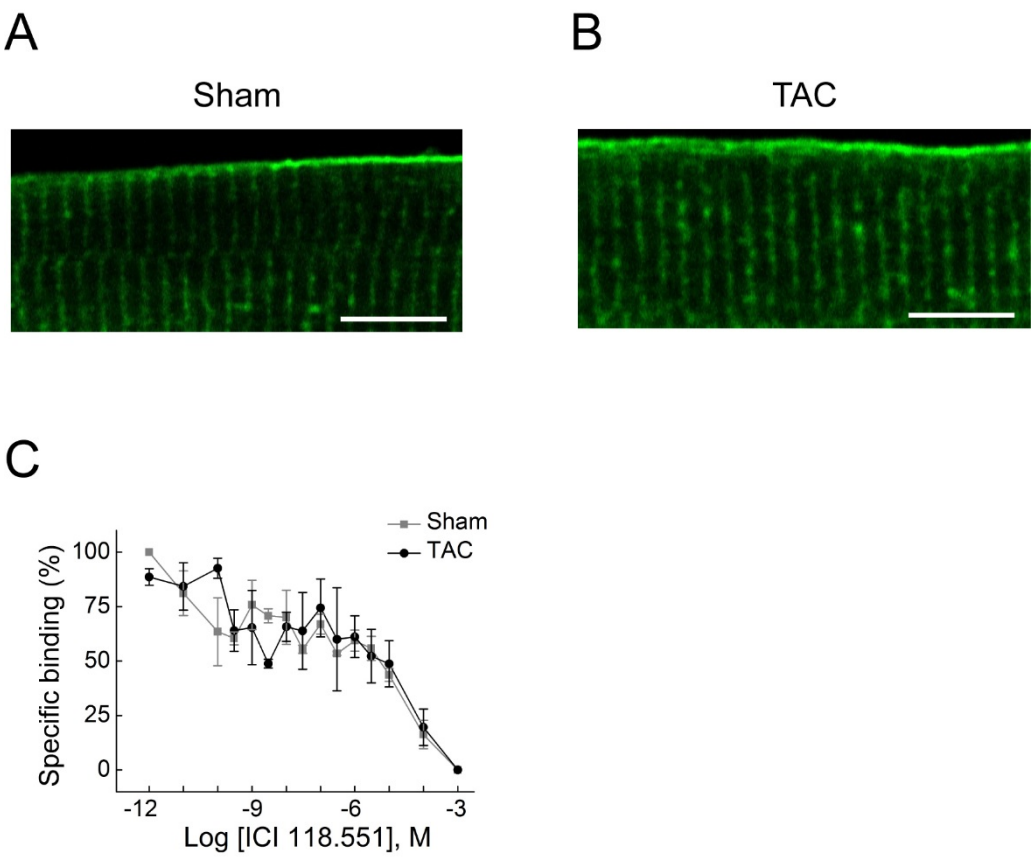
Online Figure III. *Phenotypic characterization of the pmEpac1 transgenic mouse.* Functional parameters (*A*) and heart dimensions (*B*) measured at 6 months by echocardiography are not significantly different between wildtype (WT) and transgenic (TG) littermates (n=5 mice each). PWTd, posterior wall thickness in diastole; LVIDs, left ventricular inner dimension in systole; LVIDd, left ventricular inner dimension in diastole; FS, fractional shortening; FAS, fractional area shortening; EF, ejection fraction; n.s. – not significant. (*C*) Hematoxylin-eosin staining of representative short axis cross-sections from WT and TG hearts at the ages of 3 and 6 months do not show any abnormalities. Scale bar, 2 mm. (*D*) Morphometric analysis of the heart weight to body weight (HW/BW) and heart weight to tibia length (HW/TL) ratios at 6 months of age (means ± SE, n=7 WT and 5 TG mice). (*E*) Analysis of the cardiomyocyte diameter in transverse heart sections stained with wheat germ agglutinin. Data are means ± SE, from 4 WT and 5 TG hearts with 100 cells counted in each section.



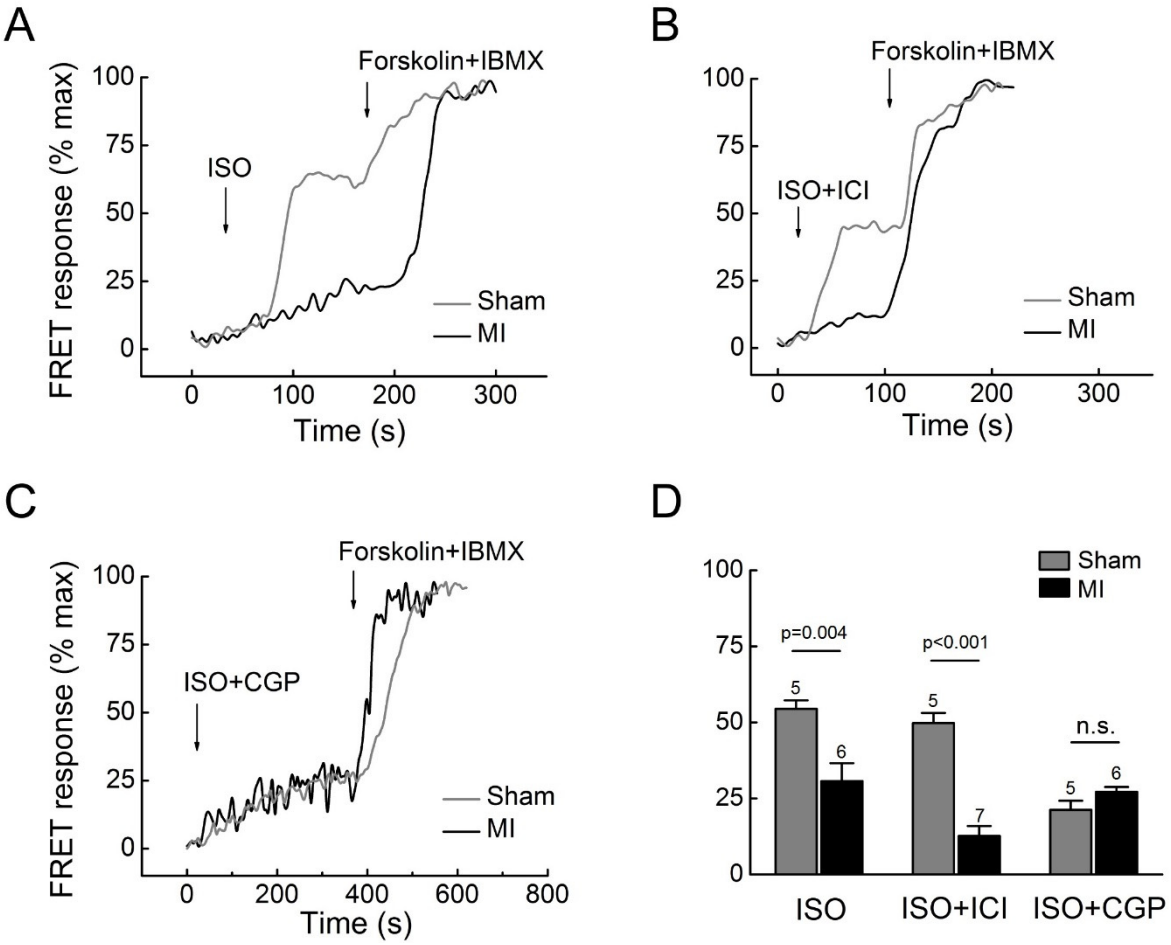
Online Figure IV. *pmEpac1 is particularly sensitive to the local β_2-AR-cAMP signals.* Representative single-cell FRET recordings with the cytosolic (Epac1-camps) and the membrane (pmEpac1) sensors. (*A*) Selective stimulation of β_1-AR with 100 nmol/L of ISO plus 50 nmol/L of the β_2-AR antagonist ICI118551 (ICI) leads to comparable cAMP signals from both sensors. Maximal stimulation was evoked by 10 µmol/L forskolin plus 100 µmol/L IBMX. (*B*) Selective β_2-AR stimulation with 100 nmol/L ISO plus 100 nmol/L of the β_1-AR blocker CGP20712A (CGP) induces a >2-fold stronger response in the membrane microdomain (*C*) Quantification of the FRET responses to ISO alone (stimulation of both β_1- and β_2-AR) and those shown in A and B, means ± SE number of cells and p-values are indicated above the bars. n.s. – not significant at p=0.05. The differences between ISO and β_1-AR responses are not significant. (*D*) Comparison of sensitivities between the two sensors using the previously established protocol.[17] Cells were pretreated with 100 µmol/L MDL-12330A for 10 min to inhibit the basal adenylyl cyclase activity and titrated with increasing concentrations of the cell-permeable cAMP analogue 8-Br-2'-OMe-cAMP-AM. EC_{50} values were 3.0 ± 1.1 and 9.1 ± 1.1 µmol/L for Epac1-camps and pmEpac1, respectively (means ± SE, n=5 and 6, respectively). Similar to previously developed membrane-targeted version of the cAMP biosensor Epac2-camps,[18] pmEpac1 shows 3-times lower affinity to cAMP than its parental cytosolic construct Epac1-camps. Epac1-camps myocytes were isolated from the previously developed transgenic mice.[19] Here and in all further experiments, we normalized the measured FRET changes to the maximal responses evoked by forskolin plus IBMX to facilitate the comparison between different conditions. This was possible because the maximal response amplitudes from untreated to fully activated state were indistinguishable in sham and TAC cells (14.7±2.1% and 15.0±2.2%, respectively, for pmEpac1 cells, means±SE, n=11-14 cells each). Of note, ISO and β_1-AR-stimulated cAMP (β_1-AR-cAMP) responses were indistinguishable, suggesting that global ISO responses are predominantly mediated by β_1-AR. Therefore, we used ISO in all subsequent experiments to stimulate this global β_1-AR-associated pool of cAMP.



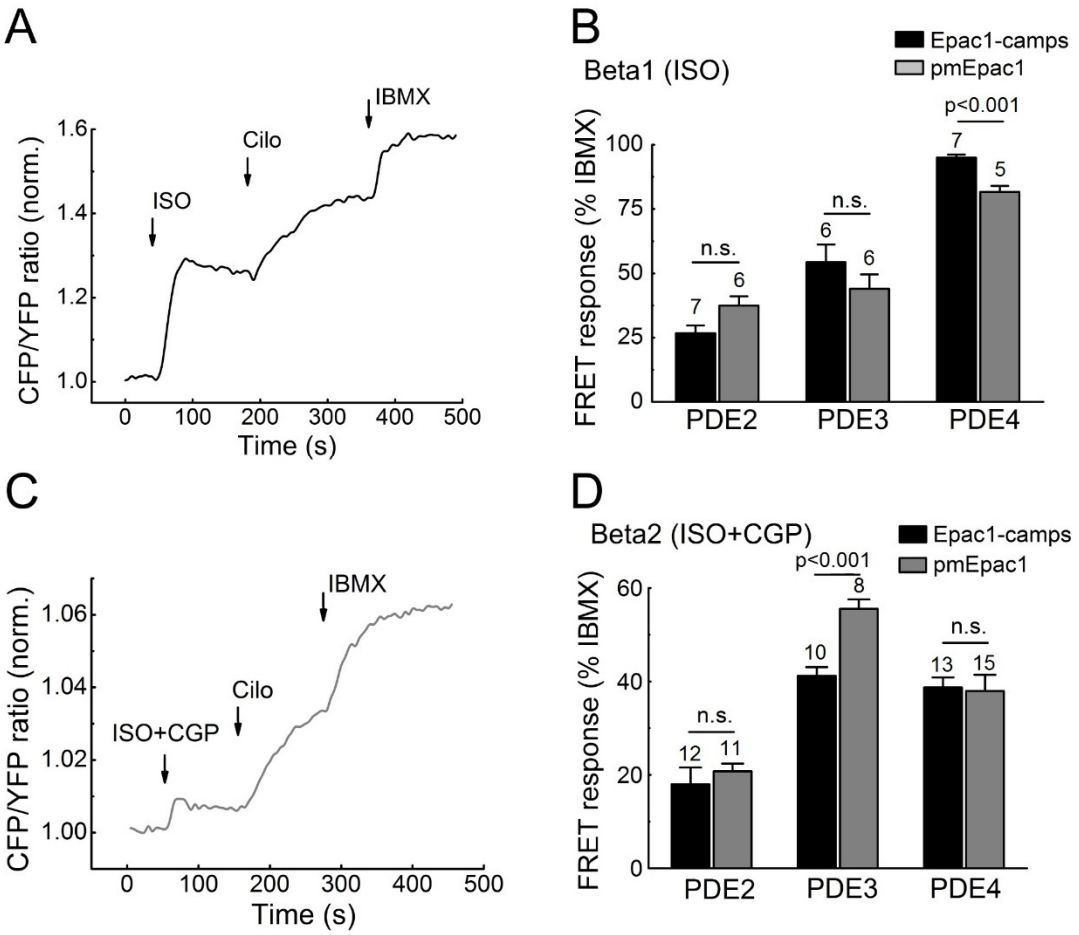
Online Figure V. *Echocardiographic parameters and plasma ANP levels in TAC compared to sham operated mice.* (*A*) Developed pressure gradients measured 3 days after aortic banding by Doppler-echocardiography. Cardiac dimensions (*B*), functional parameters (*C*), and calculated HW/BW ratios (*D*) measured 7-8 weeks after surgery. Means ± SE. Number of mice and p-values are indicated above the bars; n.s., not significant at p=0.05. Values are as described in Online Figure III. AWTd, anterior wall thickness in diastole. (*E*), ANP plasma levels in representative TAC and sham mice measured by ELISA.



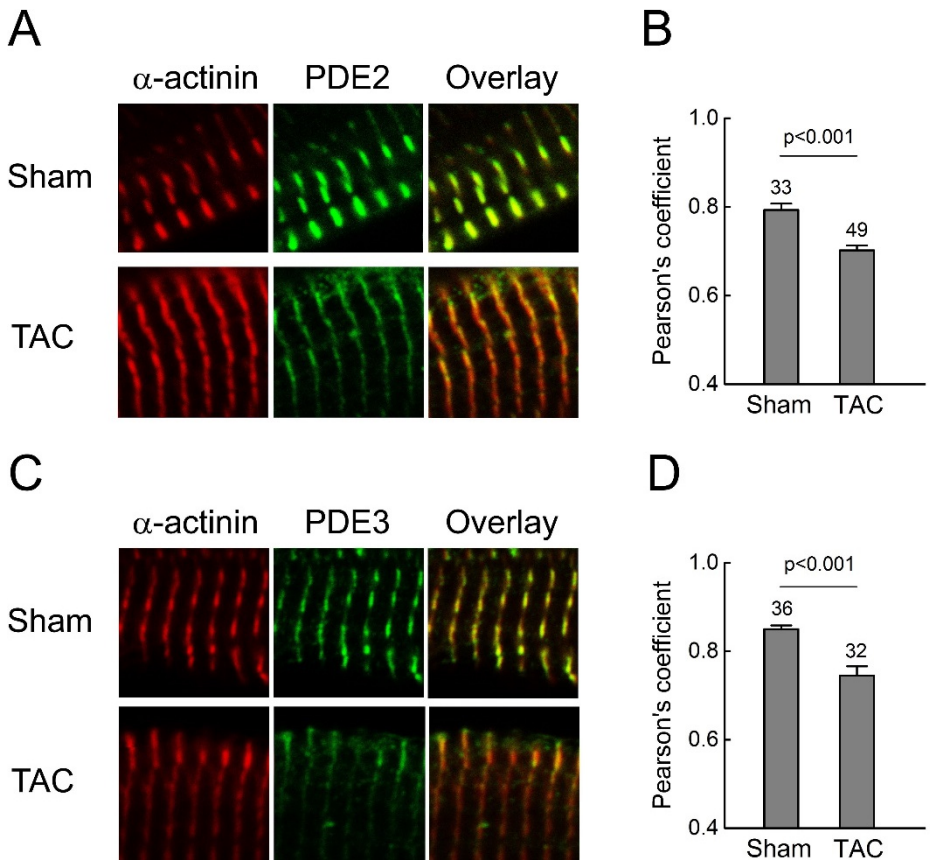
Online Figure VI. *Sensor localization and β_1/β_2-AR ratios in TAC vs sham cardiomyocytes.* (*A*,*B*) Sensor localization is virtually unaltered in diseased myocytes. Representative confocal pictures. Scale bar, 10 μM. (*C*) Radioligand (^{125}I-cyanopindolole) competition displacement curves for membranes isolated from TAC and sham myocytes. The first component of the curve represents the β_2-AR, the second phase – the β_1-AR fraction. The β_1/β_2-AR ratios are not significantly different between the groups. Data are from 3 independent experiments performed in duplicate (means ± SE).



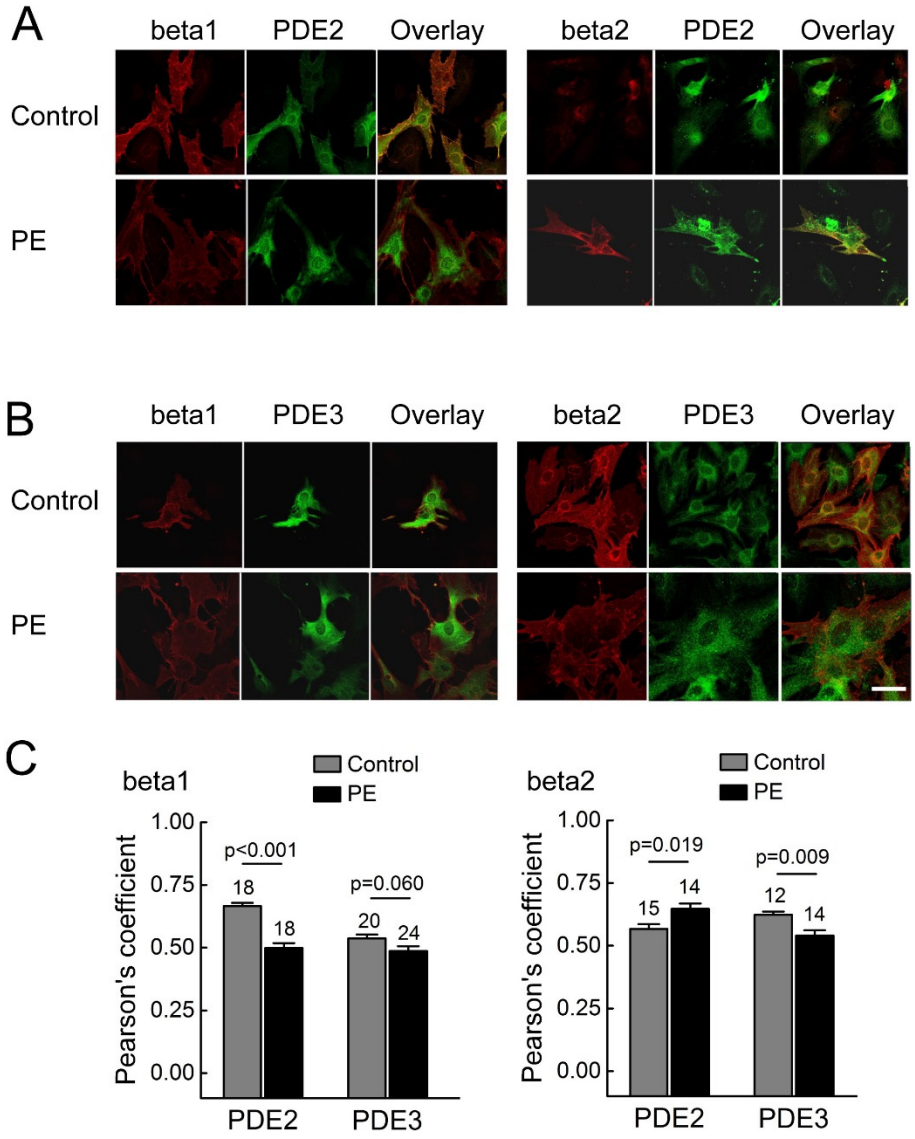
Online Figure VII. *Downregulation of β_1-AR-cAMP signalling in failing pmEpac1 cardiomyocytes. A*, Cardiomyocytes were isolated from pmEpac1 mice 12 weeks after myocardial infarction (MI) or sham surgery performed as previously described[5] to induce chronic heart phenotype which was accompanied by ~80% increase in heart-to-body weight ratio, ~60% reduction in factional shortening and a ~45% increase in left-ventricular end-diastolic internal dimension. FRET recordings during stimulation with the β-AR agonist ISO (100 nmol/L), followed by the maximal stimulation with the direct adenylyl cyclase activator forskolin (10 µmol/L) plus IBMX (100 µmol/L). (*B*, *C*) β_1- and β_2-ARs were selectively stimulated with 100 nmol/L of ISO plus 50 nmol/L of the β_2-AR antagonist ICI118551 (ICI) and with ISO plus 100 nM of the β_1-AR antagonist CGP20712A (CGP), respectively. *D*, Quantification of the FRET results shown in A-C demonstrated a pronounced β_1-AR-cAMP desensitization and unchanged β_2-AR-cAMP responses after MI. Number of cells and p-values for significant differences are indicated above the bars; n.s. – not significant at p=0.05.



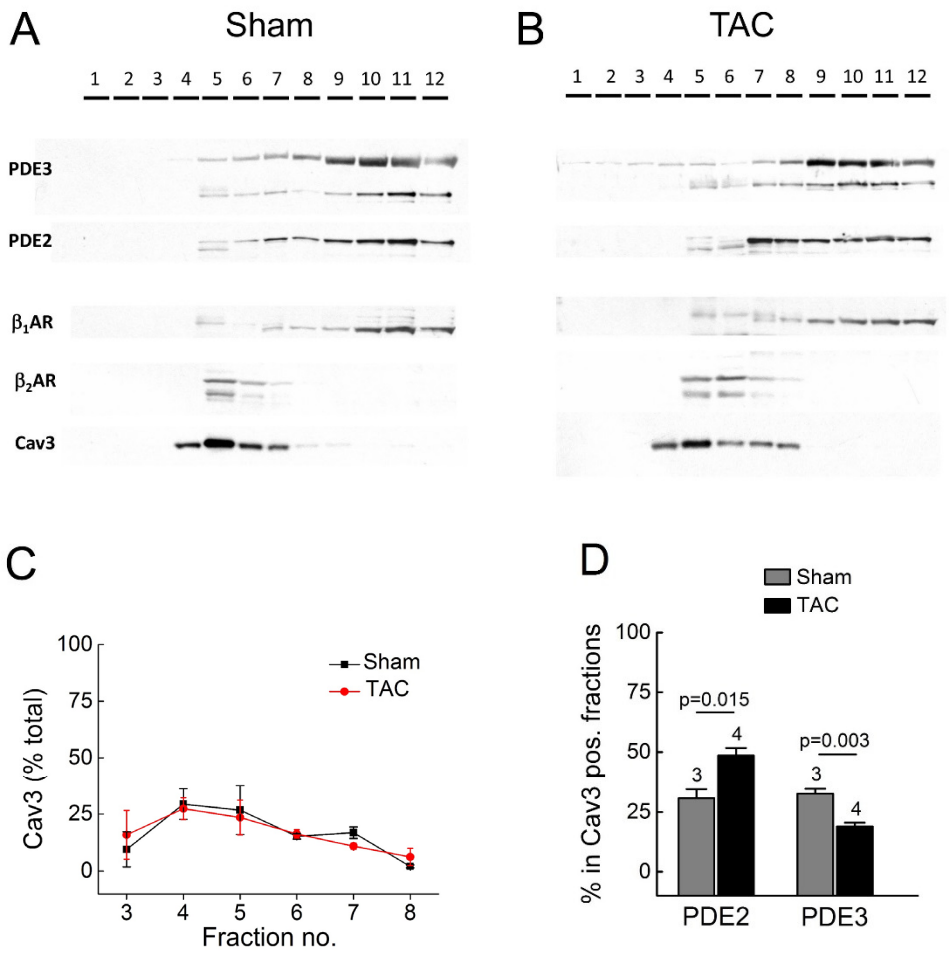
Online Figure VIII. *cAMP levels are under predominant control of PDE3 in the β_2-AR microdomain.* (*A*) Example of a FRET protocol used to evaluate contributions of individual PDE families into cAMP control in the cytosolic compartment using Epac1-camps. β_1-AR was firstly stimulated with 100 nmol/L of ISO in Epac1-camps expressing cardiomyocytes. Next, the PDE3 selective blocker cilostamide (Cilo, 10 µmol/L) was applied to further increase cAMP levels. Lastly, IBMX (100 µmol/L) was used for maximal PDE inhibitory response. (*C*) Example of a FRET protocol used to evaluate contributions of individual PDE families in receptor-associated membrane microdomains using pmEpac1. β_2-AR was prestimulated with ISO plus CGP. (*B*, *D*) Contributions of individual PDEs into the regulation of β_1- and β_2-AR-cAMP in either cytosolic (Epac1-camps) or membrane (measured with pmEpac1) microdomains, respectively, calculated as a fraction of the selective PDE-blocker signal from maximal IBMX response. Means ± SE, Number of cells and p-values for significant differences are indicated above the bars, n.s. – not significant at p=0.05. 100 mol/L BAY60-7550 and 10 µmol/L rolipram were used to fully inhibit PDE2 and PDE4, respectively. We also tested whether the cytosolic cAMP levels measured using Epac1-camps may be affected in hypertrophy. In the bulk cytosol, we could also observe similar changes in ANP effects on cAMP levels and an increase in PDE2 contribution after β_2-AR stimulation in TAC myocytes, while changes in PDE3 effects were somewhat less pronounced (Online Figure XV A,B). The increased effect of ANP applied after ISO in TAC myocytes also correlated with phosphorylation of PKA substrates involved in the regulation of contractility such as phospholamban and myosin binding protein C (see Online Figure XVC).



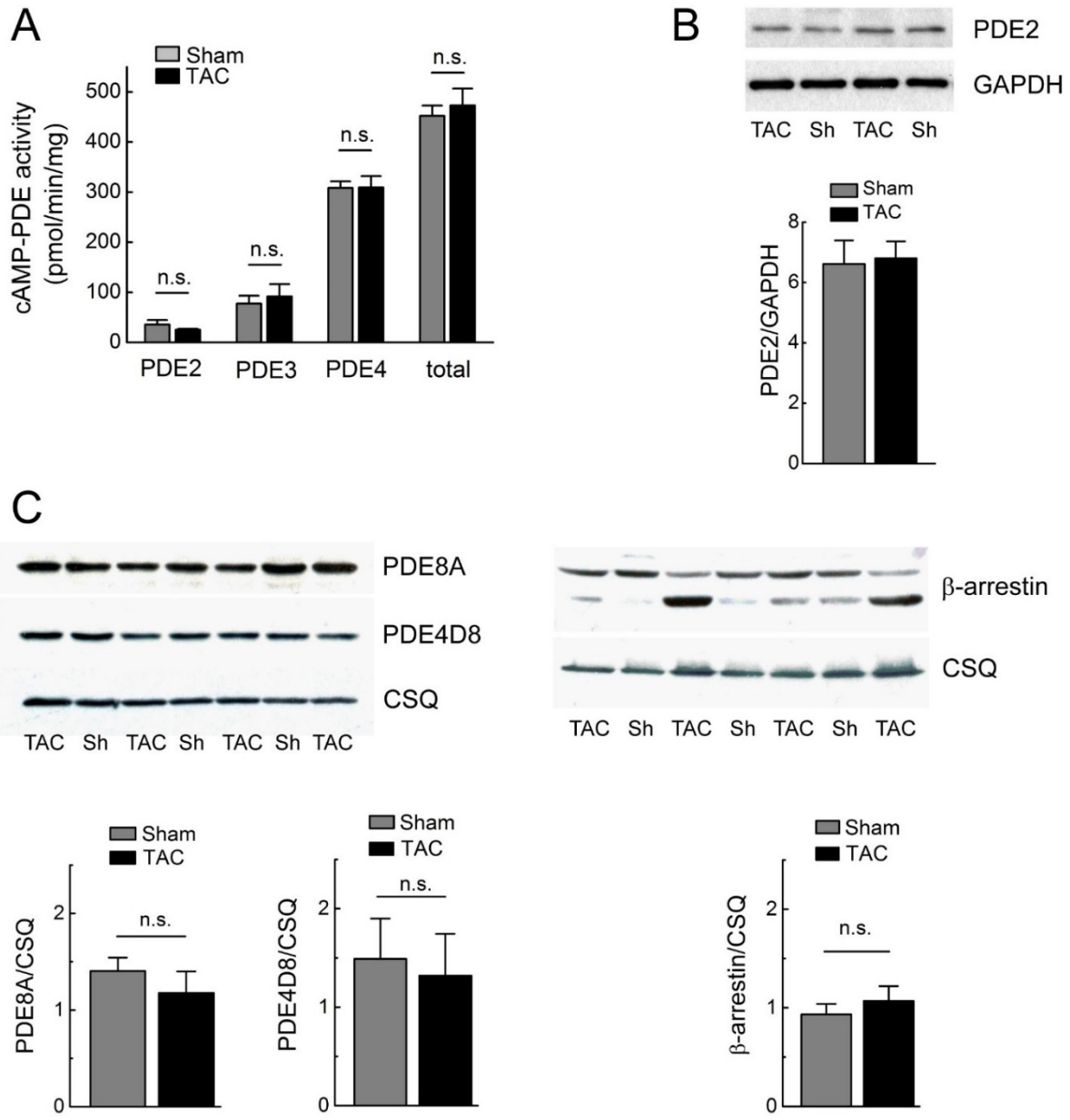
Online Figure IX. *Confocal microscopy analysis of PDE2 and PDE3 localization.* Sham and TAC myocytes were immunostained with the α-actinin and PDE2A (*A*) or PDE3A (*B*) family-specific antibodies. High degree of co-localisation quantitated by Pearson's coefficient is significantly reduced in hypertrophic cells. Data are means ± SE from 4 sham and 5 TAC mice each, number of cells analyzed and p-values are indicated above the bars.



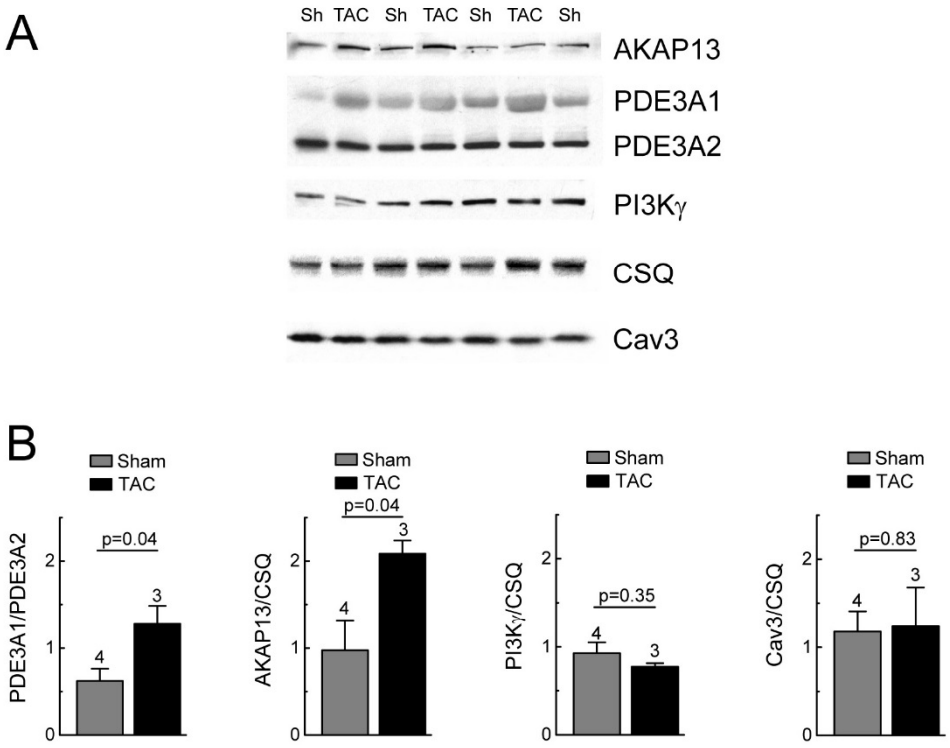
Online Figure X. *Redistribution of PDE2 and PDE3 in neonatal cardiomyocyte hypertrophy model.* Neonatal rat myocytes were transduced with adenoviruses expressing HA-β_1-AR or FLAG-β_2-AR and stimulated for 48 h with 50 µmol/L phenylephrine (PE) to induce hypertrophy. Cells were fixed and co-stained for either PDE2 (A) or PDE3 (B) and affinity-tagged receptors. *(C)* Co-localization was quantitated by Pearson's coefficient. Data are means ± SE, number of analyzed cells and p-values are indicated above the bars.



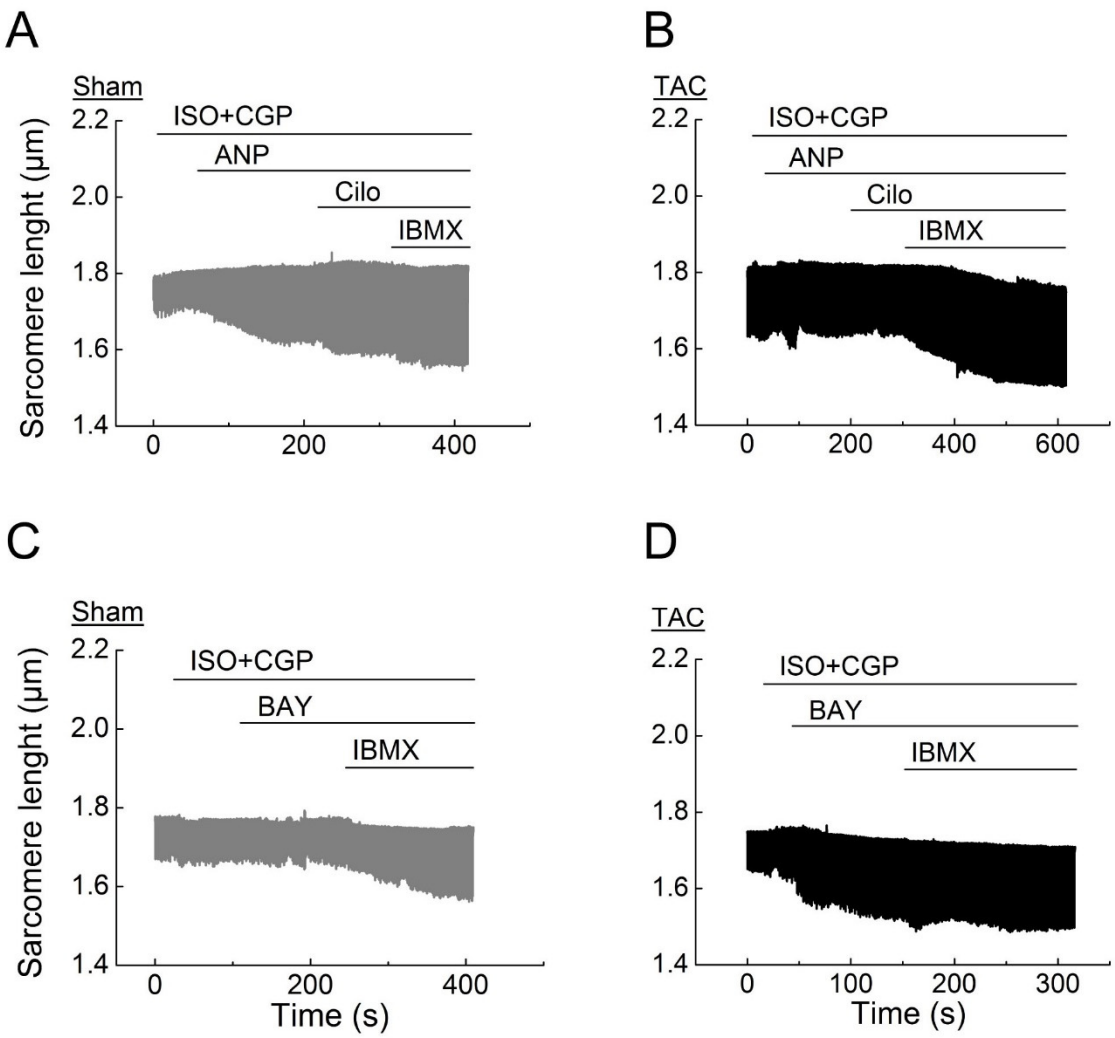
Online Figure XI. *Subcellular fractionation of sham and TAC hearts.* Heart lysates were subjected to density gradient fractionation, and individual fractions (1-12) were immunoprobed with available antibodies for β_1-AR, β_2-AR, PDE2, PDE3 and caveolin 3 (Cav3). Representative immunoblots for sham (*A*) and TAC (*B*) hearts are presented. *(C)*, Distribution of Cav3 between the fractions averaged over all analyzed Sham and TAC hearts shows no significant Cav3 movement/redistribution in our disease model. Shown is a proportion of Cav3 immunoreactivity in each individual fraction as % of total Cav3 amount detected in all fractions. Means ± SE, n=3 Sham and 4 TAC hearts *(D)*, Quantification of PDE2 and PDE3 amounts associated with caveolin-positive fractions. Means ± SE, number of hearts and p-values are indicated above the bars.



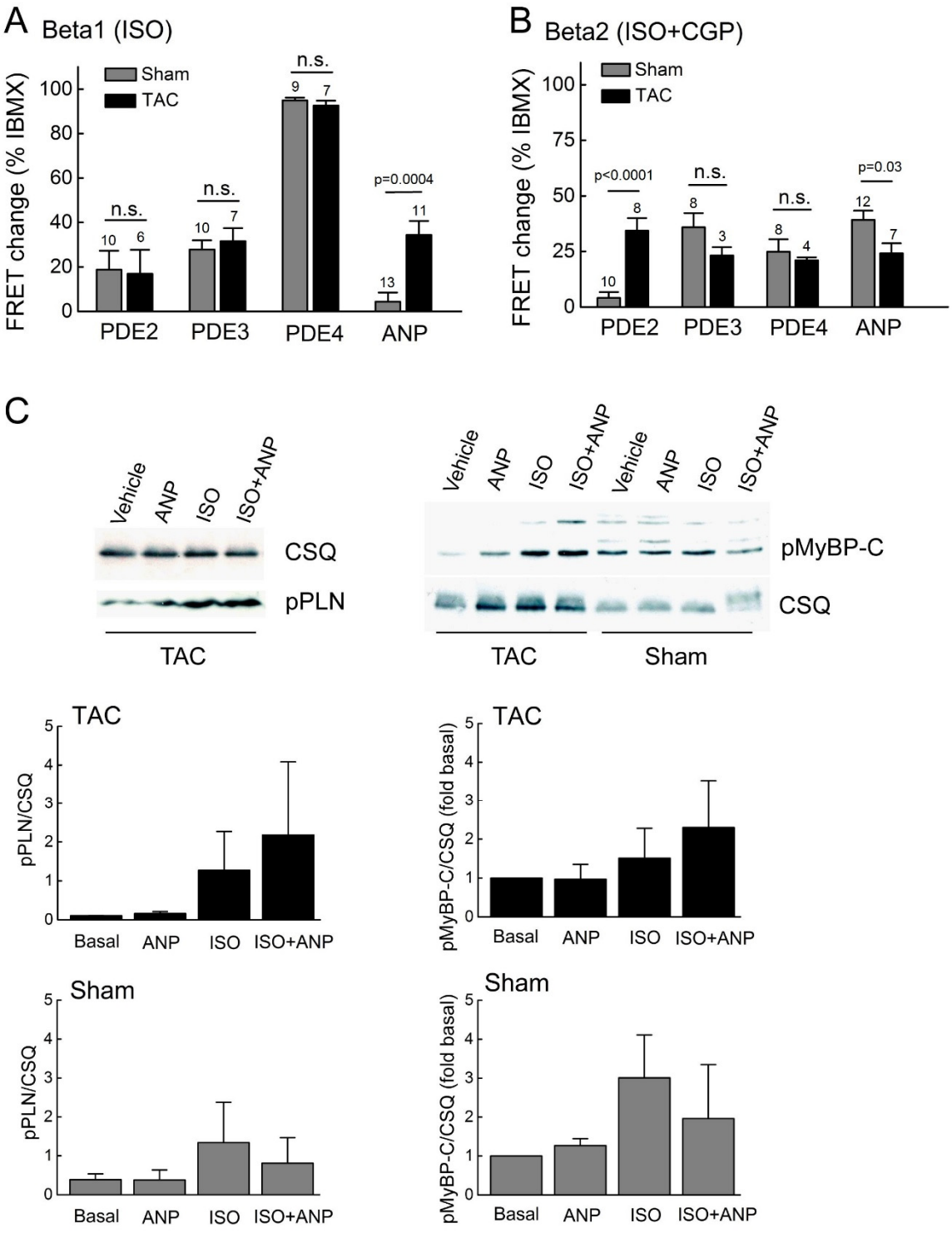
Online Figure XII. *Expression and activities of the cAMP-hydrolysing phosphodiesterases (PDEs) in TAC and sham (Sh) cardiomyocytes.* (*A*) cAMP-PDE activities measured in cardiomyocyte lysates using *in vitro* assays were not significantly different between the groups (n=3 sham and 4 TAC hearts). (*B*) Immunoblot analysis of PDE2, protein expression levels in TAC and sham cells, means ± SE, n = 3 each, n.s. – not significant. (*C*) Protein expression levels of PDE8A, PDE4D8 and of β-arrestin normalized to calsequestrin (CSQ) were also unchanged. Shown are means ± SE, n = 3 sham and 4 TAC, n.s. – not significant.



Online Figure XIII. *Expression of PDE3A isoforms, scaffolding proteins AKAP13 (AKAP-Lbc) and PI3Kγ, as well as of caveolin 3 (Cav3) in sham (Sh) and TAC hearts.* Representative immunoblots (*A*) and their analysis (*B*) are shown. Early hypertrophy model is associated with an increase of AKAP-Lbc expression and an increased PDE3A1/PDE3A2 isoform ratio. Shown are means ± SE, number of hearts and p-values are indicated above the bars.



Online Figure XIV. *Switch from PDE3 to PDE2 abrogates the positive ANP effect on β_2-AR-stimulated cardiomyocyte contractility in hypertrophied cells.* (*A* and *B*) Measurements of sarcomere shortening in healthy vs. diseased cells selectively stimulated with β_2-AR ligands (ISO+CGP) and subsequently treated with ANP (100 nmol/L), cilostamide (Cilo, 10 µmol/L) and IBMX (100 µmol/L). Positive effects of ANP and cilostamide on the contractile force after β_2-AR stimulation are abolished in diseased cells. (*C* and D) BAY60-7550 (BAY, 100 nmol/L) after β_2-AR stimulation leads to an increase in contractility only in TAC cardiomyocytes. Data analysis is shown in Figure 4E. As shown previously,[10] β_2-AR stimulation induced a slight positive inotropic effect (see Figure 4E). Similar to our FRET results, ANP and cilostamide further increased the contractile response measured by sarcomere shortening after β_2-AR stimulation, and the positive effect of ANP or cilostamide applied after ISO on contractility was completely abolished in hypertrophied cells



Online Figure XV. *Measurements of PDE and ANP effects in the cytosol of hypertrophied myocytes.* (*A,B*) Effects of PDE family inhibitors and of ANP were measured in the bulk cytosol of Epac1-camps transgenic cells after ISO and β_2-AR selective prestimulation exactly as described in Figure 3 and Online Figure XIV, respectively. Number of cells and p-values for significant differences are indicated above the bars; n.s. – not significant at p=0.05. *(C)* Immunoblot analysis of PKA substrate phosphorylation upon ISO and ISO+ANP treatment. Shown are representative immunoblots for phospho-phospholamban (pPLN, Ser16) and phospho-myosin binding protein C (pMyBP-C, Ser282) and quantifications from 3 independent experiments (means ± SE). These data obtained with less sensitive classical biochemical methods show only a tendency for augmented phosphorylation after ANP treatment in TAC cells.

Supplemental References

1. Zacharias DA, Violin JD, Newton AC, Tsien RY. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science*. 2002;296:913-916
2. Nikolaev VO, Bunemann M, Hein L, Hannawacker A, Lohse MJ. Novel single chain cAMP sensors for receptor-induced signal propagation. *J Biol Chem*. 2004;279:37215-37218
3. Nikolaev VO, Bunemann M, Schmitteckert E, Lohse MJ, Engelhardt S. Cyclic AMP imaging in adult cardiac myocytes reveals far-reaching beta1-adrenergic but locally confined beta2-adrenergic receptor-mediated signaling. *Circ Res*. 2006;99:1084-1091
4. Buitrago M, Lorenz K, Maass AH, Oberdorf-Maass S, Keller U, Schmitteckert EM, Ivashchenko Y, Lohse MJ, Engelhardt S. The transcriptional repressor Nab1 is a specific regulator of pathological cardiac hypertrophy. *Nat Med*. 2005;11:837-844
5. Wagner E, Lauterbach MA, Kohl T, Westphal V, Williams GS, Steinbrecher JH, Streich JH, Korff B, Tuan HT, Hagen B, Luther S, Hasenfuss G, Parlitz U, Jafri MS, Hell SW, Lederer WJ, Lehnart SE. Stimulated emission depletion live-cell super-resolution imaging shows proliferative remodeling of T-tubule membrane structures after myocardial infarction. *Circ Res*. 2012;111:402-414
6. Hu P, Zhang D, Swenson L, Chakrabarti G, Abel ED, Litwin SE. Minimally invasive aortic banding in mice: effects of altered cardiomyocyte insulin signaling during pressure overload. *Am J Physiol Heart Circ Physiol*. 2003;285:H1261-1269
7. Gorelik J, Yang LQ, Zhang Y, Lab M, Korchev Y, Harding SE. A novel Z-groove index characterizing myocardial surface structure. *Cardiovasc Res*. 2006;72:422-429
8. Pacher P, Nagayama T, Mukhopadhyay P, Batkai S, Kass DA. Measurement of cardiac function using pressure-volume conductance catheter technique in mice and rats. *Nat Protoc*. 2008;3:1422-1434
9. Grebe C, Klingebiel TM, Grau SP, Toischer K, Didie M, Jacobshagen C, Dullin C, Hasenfuss G, Seidler T. Enhanced expression of DYRK1A in cardiomyocytes inhibits acute NFAT activation but does not prevent hypertrophy in vivo. *Cardiovasc Res*. 2011;90:521-528
10. Paur H, Wright PT, Sikkel MB, Tranter MH, Mansfield C, O'Gara P, Stuckey DJ, Nikolaev VO, Diakonov I, Pannell L, Gong H, Sun H, Peters NS, Petrou M, Zheng Z, Gorelik J, Lyon AR, Harding SE. High levels of circulating epinephrine trigger apical cardiodepression in a beta2-adrenergic receptor/Gi-dependent manner: a new model of Takotsubo cardiomyopathy. *Circulation*. 2012;126:697-706
11. Thompson WJ, Appleman MM. Multiple cyclic nucleotide phosphodiesterase activities from rat brain. *Biochemistry*. 1971;10:311-316
12. Richter W, Conti M. Dimerization of the type 4 cAMP-specific phosphodiesterases is mediated by the upstream conserved regions (UCRs). *J Biol Chem*. 2002;277:40212-40221
13. Head BP, Patel HH, Roth DM, Lai NC, Niesman IR, Farquhar MG, Insel PA. G-protein-coupled receptor signaling components localize in both sarcolemmal and intracellular caveolin-3-associated microdomains in adult cardiac myocytes. *J Biol Chem*. 2005;280:31036-31044
14. Klaiber M, Dankworth B, Kruse M, Hartmann M, Nikolaev VO, Yang RB, Volker K, Gassner B, Oberwinkler H, Feil R, Freichel M, Groschner K, Skryabin BV, Frantz S, Birnbaumer L, Pongs O, Kuhn M. A cardiac pathway of cyclic GMP-independent signaling of guanylyl cyclase A, the receptor for atrial natriuretic peptide. *Proc Natl Acad Sci U S A*. 2011;108:18500-18505
15. Jäger R, Groneberg D, Lies B, Bettaga N, Kummel M, Friebe A. Radioimmunoassay for the quantification of cGMP levels in cells and tissues. *Methods Mol Biol*. 2013;1020:63-72

16. Kuhn M. Structure, regulation, and function of mammalian membrane guanylyl cyclase receptors, with a focus on guanylyl cyclase-A. *Circ Res*. 2003;93:700-709
17. Börner S, Schwede F, Schlipp A, Berisha F, Calebiro D, Lohse MJ, Nikolaev VO. FRET measurements of intracellular cAMP concentrations and cAMP analog permeability in intact cells. *Nat Protoc*. 2011;6:427-438
18. Wachten S, Masada N, Ayling LJ, Ciruela A, Nikolaev VO, Lohse MJ, Cooper DM. Distinct pools of cAMP centre on different isoforms of adenylyl cyclase in pituitary-derived GH3B6 cells. *J Cell Sci*. 2010;123:95-106
19. Calebiro D, Nikolaev VO, Gagliani MC, de Filippis T, Dees C, Tacchetti C, Persani L, Lohse MJ. Persistent cAMP-signals triggered by internalized G-protein-coupled receptors. *PLoS Biol*. 2009;7:e1000172

Circulation Research
JOURNAL OF THE AMERICAN HEART ASSOCIATION

Microdomain Switch of cGMP-Regulated Phosphodiesterases Leads to ANP-Induced Augmentation of β-Adrenoceptor-Stimulated Contractility in Early Cardiac Hypertrophy

Ruwan K Perera, Julia Sprenger, Julia H Steinbrecher, Daniela Hübscher, Stephan E Lehnart, Marco Abeßer, Kai Schuh, Ali El-Armouche and Viacheslav O Nikolaev

Circ Res. published online February 16, 2015;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7330. Online ISSN: 1524-4571