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


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–associated cAMP and cGMP microdomain signaling in early cardiac disease.

Methods and Results: Unexpectedly, measurements of cell shortening revealed augmented β-adrenergic receptor–stimulated cardiomyocyte contractility by atrial natriuretic peptide/cGMP signaling in early cardiac hypertrophy after 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 β1- and β2-adrenergic receptor–associated membrane microdomains using a novel membrane-targeted Förster resonance energy transfer–based biosensor transgenically expressed in mice revealed that this unexpected atrial natriuretic peptide 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. (Circ Res. 2015;116:1304-1311. DOI: 10.1161/CIRCRESAHA.116.306082.)

Key Words: 3',5'-cyclic-AMP phosphodiesterases ■ atrial natriuretic factor ■ membrane microdomains ■ phosphodiesterase 3 inhibitors ■ receptors, adrenergic

The cyclic nucleotides, cAMP and 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, its anchoring proteins, and cAMP/cGMP hydrolyzing enzymes phosphodiesterases involved in termination and spatial confinement of cyclic nucleotide signaling.1,2 cGMP can regulate compartmentalized cAMP signaling by either stimulating phosphodiesterase 2 or inhibiting phosphodiesterase 3 activity.3,4 However, because of 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.

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Two major subtypes of cardiac β-adrenergic receptors (β-ARs) modulate the effects of catecholamines on excitation–contraction coupling. The predominantly G1-coupled β1-AR is evenly distributed across the sarcolemma and stimulates...
far-reaching cAMP signals, which strongly increase contractility and promote cardiac remodeling. In contrast, the β2-AR couples to both Gs- and Gi-proteins and is exclusively located in transverse-tubules, thereby generating highly localized cAMP responses, which may protect from apoptosis and pathological remodeling.5,6 cGMP has been reported as a cardioprotective second messenger, because stimulation of cGMP production or inhibition of its degradation by cGMP-phosphodiesterase inhibitors leads to a reduction of β2-AR–stimulated pathological hypertrophy.7 Conversely, genetic deletion of the atrial natriuretic peptide (ANP) or brain natriuretic peptide receptor guanylyl cyclase A (GC-A) promotes cardiac hypertrophy.8,9 One well-established hallmark of chronic cardiac disease is the dramatic reduction of β1-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 and 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 phosphodiesterase 2/3 between the β1- and β2-AR–associated microdomains without any change of whole-cell phosphodiesterase 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 in the Online Data 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

All animal experiments were performed according to the institutional and governmental guidelines as described.13 Mice were euthanized 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 1-way ANOVA or Mann–Whitney tests, followed by Bonferroni post hoc test. Data are presented as means±SE from the indicated number of independent experiments (animals and cells) per condition.

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Results

ANP Increases Contractility in Hypertrophied Cardiomyocytes and Hearts

ANP has previously been found not to affect the isoproterenol-stimulated contractility of single healthy mouse ventricular cardiomyocytes.14 Accordingly, ANP did not significantly change sarcomere shortening after submaximal isoproterenol stimulation of healthy cells (Figure 1A and 1C). However, in cardiomyocytes isolated from hearts after transverse aortic constriction (TAC), ANP applied after isoproterenol but not alone strikingly led to a significant positive inotropic response (Figure 1B and 1C). This effect could be mimicked by a cell-permeable cGMP analogue cGMP-AM but not by the selective NPR-C (clearance) receptor agonist cANP(4–23) (Online Figure IA, Online Data Supplement), 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 isoproterenol prestimulation (Figure 1D).

This finding could be also confirmed by hemodynamic measurements in vivo, where ANP augmented isoproterenol-induced increase of heart rate and showed a strong tendency for an increased cardiac output (Figure 1E and 1F). Remarkably, the ANP-stimulated cGMP levels were not reduced in TAC cells (Online Figure IB), suggesting the absence of GC-A desensitization. This behavior of hypertrophied cardiomyocytes, which is in contrast to well-established β-AR and ANP receptor desensitization11,15 and absence of ANP effect on contractility occurring in chronic heart failure16,17 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

Because 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 β2-AR CAMP signals (Online Figure IV), which are known to compartmentalize close to transverse-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
transverse-tubular membranes and surface sarcolemma was virtually unchanged (Online Figure VIA and VIB). 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 isoproterenol (3 nmol/L) and next applied 100 nmol/L of ANP. Indeed, ANP led to augmentation of isoproterenol-stimulated cAMP levels exclusively in TAC cells (Figure 2A and 2B). To further test how cAMP levels are affected at saturating β-AR agonist concentrations and which receptor subtypes are involved, we compared β₁- and β₂-AR specific responses in TAC versus sham cells and found significantly enhanced global isoproterenol responses, which were because of stronger β₁-AR signals (Figure 2C, 2D, and 2F). In contrast, β₂-AR–stimulated cAMP (β₂-AR cAMP) responses were significantly reduced in diseased cells (Figure 2E and 2F). These changes of β-AR-cAMP signals were not because of altered β-AR densities (164±16 versus 128±17 fmol/mg membrane protein in sham versus TAC cells, not significant, P=0.1, n=3) or β₁/β₂-AR ratio, as confirmed by radioligand binding studies.
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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 β1-AR-cAMP signaling with unchanged β2-AR responses (Online Figure VII).

**In Hypertrophy, cGMP-Sensitive Phosphodiesterases Redistribute Between β₁-AR–Associated and β₂-AR–Associated Membrane Microdomains**

We next studied the effects of various phosphodiesterase inhibitors after global and β₂-AR–selective receptor stimulations in healthy cells. We found that although the β₁-AR-cAMP microdomain is mostly controlled by phosphodiesterase 4 (followed by less active phosphodiesterase 3 and phosphodiesterase 2), the β₂-AR-cAMP microdomain is under predominant control of phosphodiesterase 3, 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, phosphodiesterase 2 effects after global β₁-AR stimulation were significantly reduced, whereas phosphodiesterase 3 and 4 inhibitor responses remained unchanged (Figure 3A–3C). In contrast, the predominant regulation of β₂-AR-cAMP by phosphodiesterase 3 in this microdomain
Fig. 3. Phosphodiesterase 2 and phosphodiesterase 3 redistribute between β1- and β2-adrenergic receptor (AR)-associated cAMP microdomains in cardiac hypertrophy. (A and B) Representative traces showing cilostamide (Cilo, 10 μmol/L) and BAY60-7550 (BAY, 100 nmol/L) effects in transverse aortic constriction (TAC) and sham pmEpac1 cardiomycocytes after global isoproterenol (ISO) stimulation. C. Quantifications of the phosphodiesterase inhibitor signals (as a percentage of the maximal response evoked by the unselective inhibitor blocker 3-Isobutyl-1-methylxanthine (IBMX) obtained after ISO stimulation in TAC and sham cells. D. After selective β1-AR (ISO+CGP) pretimulation, phosphodiesterase 3 inhibitor effects are dramatically reduced while phosphodiesterase 2 inhibitor responses are markedly increased in hypertrophied cells (E). F. Quantifications of the phosphodiesterase inhibitor signals after β1-AR selective stimulation. Shown are means±SE, number of cells and P values for significant differences are indicated above the bars.

was abolished. Instead, the phosphodiesterase 2 effects were significantly increased, whereas phosphodiesterase 4 effects remained unchanged (Figure 3D–3F). These findings suggest a redistribution of phosphodiesterase 2 from the β1-AR to the β2-AR-associated microdomains and a selective downregulation of phosphodiesterase 3 in the β1-AR compartment. To test whether the disease leads to changes in physical localization of phosphodiesterase 2 and 3, we stained the cells with phosphodiesterase family-specific antibodies and analyzed the degree of their colocalization with α-actinin. Interestingly, TAC led to measurable alterations in subcellular phosphodiesterase 2 and 3 distribution (Online Figure IX). Furthermore, relocation of phosphodiesterase 2 between β1-AR and β2-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 phosphodiesterase 2 from β1-AR–associated noncaveolar into β2-AR–containing caveolar fractions in disease, whereas phosphodiesterase 3 proportion in caveolin-positive fractions was reduced (Online Figure XI). These data directly support our hypothesis that altered phosphodiesterase 2/3 inhibitor effects indeed arise from subcellular recolocalization of these phosphodiesterases.

Previously, it has been shown that under advanced cardiac hypertrophy, phosphodiesterases 3 and 4 activities are downregulated in rodent and human cardiomyocytes,18,19 whereas phosphodiesterase 2 activity (which is a crucial phosphodiesterase 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 phosphodiesterases 2 to 4 activities were unchanged (Online Figure XIII). Likewise, net expression levels of phosphodiesterase 2 analyzed by immunoblotting with whole-cell lysates were unaltered (Online Figure XIIIB). In addition, protein levels of the phosphodiesterase 4D8 isoform, which directly interacts with β1-AR,22 or of β-arrestin 2, which recruits phosphodiesterases to the β2-AR, and of the functionally-relevant 3-isobutyl-1-methylxanthine–insensitive phosphodiesterase 8A23 were also unaffected (Online Figure XIIIC). Collectively, these data indicate an early subcellular remodeling of phosphodiesterases and a switch between 2 cGMP-regulated phosphodiesterases in the β2-AR microdomain, that is, increase in local cGMP-stimulated phosphodiesterase 2 and a decrease in cGMP-inhibited phosphodiesterase 3 activities, occurring without any change of global phosphodiesterase activity and content at the cellular level. Interestingly, using immunoblot analysis we could detect a disease-associated shift of phosphodiesterase 3A isoforms from the primarily expressed phosphodiesterase 3A2 to the longest phosphodiesterase 3A1 isoform (Online Figure XIII), which is localized predominantly at sarcoplasmic reticulum instead of the plasma membrane.24 Another potential mechanism of different phosphodiesterase localization might be an altered expression of scaffolding proteins,1 of which AKAP13 (AKAP-Lbc) was increased, whereas PI3γ kinase was unchanged in our disease model (Online Figure XIII).

cGMP-Dependent Regulation of β2-AR-cAMP Signals Is Altered in Hypertrophy

To study whether the altered balance between phosphodiesterases 2 and 3 in hypertrophy contributes to the cGMP-dependent regulation of cAMP levels in the β2-AR microdomain, we measured the effect of ANP in TAC and sham pmEpac1 cardiomyocytes after selective β2-AR stimulation. Addition of ANP could further enhance the β2-AR–prestimulated cAMP levels in healthy cells, which was because of cGMP-mediated phosphodiesterase 3 inhibition, because this effect was abolished in the presence of the phosphodiesterase 3–selective inhibitor cilostamide (Figure 4A and 4B). In diseased cardiomyocytes, the ANP effect on β2-AR-cAMP levels was dramatically reduced. However, pretreatment of cells with
the phosphodiesterase 2–selective inhibitor BAY-60-7550 (BAY) could fully reverse this effect (Figure 4C and 4D), suggesting that in cardiac hypertrophy, the redistribution of phosphodiesterases 2 and 3 inverses the cGMP-mediated effects on the β2-AR-cAMP pool. The described cGMP-dependent effects on β2-AR-cAMP did also directly translate into contractile responses. 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 phosphodiesterase 2 from the β1- to the β2-AR-associated membrane microdomains leads to a decrease of the local β2-AR-cAMP and to an increase of global β1-AR-cAMP pool under elevated ANP and cGMP levels observed in early disease. The switch of β2-AR-associated phosphodiesterase 2 and 3 leads to a turnaround of cGMP/cAMP cross-talk between the β1- and β2-AR-associated microdomains, through which elevated ANP augments β-AR-adrenoceptor-stimulated contractility under shifted β1/β2-AR-cAMP balance. NE indicates norepinephrine, the physiological β-AR agonist. FRET indicates Förster resonance energy transfer; and IBMX, blocker 3-Isobutyl-1-methylxanthine.
cell contractile responses (Online Figure XIV A and XIV B and 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 phosphodiesterases 2 and 3 redistribution between receptor-associated microdomains. Interestingly, cGMP-AM, which directly acts on these redistributed phosphodiesterases, could also here recapitulate the effect of ANP. Therefore, 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 β2-AR to the β1-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 phosphodiesterase 2 and 3 between the β1-AR and β2-AR–associated membrane microdomains. This redistribution leads to switching of cGMP/cAMP cross-talk from the ANP/cGMP/phosphodiesterase 3–mediated cAMP increase to an ANP/cGMP/phosphodiesterase 2–mediated cAMP decrease after β2-AR stimulation, and exactly the opposite effect after β1-AR stimulation (Figure 4F). Functionally, not only does this lead to a change in physiologically less relevant β2-AR–specific contractile effects, but strikingly also to stimulatory ANP-effects on global contractile force after β2-AR stimulation in hypertrophied myocytes. This effect is apparently caused by a shift in β1/β2-AR signal balance toward β1-AR signaling. Mechanistically, ANP-mediated potentiation of β2-AR inotropic response at this stage of disease originates from the reorganization of microdomain-specific phosphodiesterase activity patterns that cause a shift of ANP-driven cAMP augmenting effect from local (β2-AR) toward global (β1-AR) cAMP pools. Unlike pronounced β1-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 seem rather neutral with regard to the whole-cell phosphodiesterase activities, yet we show that even slight changes in local phosphodiesterase 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 and 1C and 4F). Importantly, the disease-relevant GC-A ligands natriuretic peptides ANP and brain natriuretic peptide 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 β2-AR, which generally plays a minor role in catecholamine-induced inotropic response, generates an important signaling microdomain, which can be regulated by either phosphodiesterase 2 or phosphodiesterase 3 to exert opposite effects on global β-AR–mediated contractility by the altered functional balance between the β1- and β2-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=β1-AR-cAMP/β2-AR-cAMP) in hypertrophy.

In summary, we uncovered a new adaptation mechanism in early cardiac disease, which involves subcellular redistribution of cGMP-regulated phosphodiesterases. We propose that such alterations well precede well-known cellular remodeling, which occurs during decompensated hypertrophy and heart failure associated with changed whole-cell phosphodiesterase activities and the desensitization of the β2-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.

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Disclosures

None.

References

In contrast to chronic disease, early cardiac hypertrophy is associated with chronic decompensated cardiac disease, such as heart failure. Cyclic nucleotides, cAMP and cGMP, play crucial roles in regulating cardiac contractile function. A transgenic mouse expressing a highly-sensitive membrane-targeted Förster resonance energy transfer biosensor for cAMP and cGMP analog analog permeability in intact cells. Novelty and Significance

What Is Known?

- Cyclic nucleotides, cAMP and cGMP, play crucial roles in regulating cardiac contractile function by acting in discrete subcellular microdomains.
- Chronic decompenated cardiac disease, such as heart failure, is associated with attenuated cAMP and cGMP signaling from β-adrenergic (β-AR) and natriuretic peptide receptors, respectively.

What New Information Does This Article Contribute?

- A novel transgenic mouse, which expresses a highly-sensitive Förster resonance energy transfer-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 β1-AR induced force (in vitro) and frequency (in vitro and in vivo) of contraction because of a redistribution of cGMP-regulated phosphodiesterases 2 and 3 between the β1- and β2-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.
Microdomain Switch of cGMP-Regulated Phosphodiesterases Leads to ANP-Induced Augmentation of β-Adrenoceptor-Stimulated Contractility in Early Cardiac Hypertrophy

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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

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Detailed Methods

Cloning and transgenic mouse generation. DNA encoding the N-terminal 10 amino acid peptide MGSINSKRKD from the Lyn kinase was subcloned upstream of the start ATG of the Epac1-camps sensor 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. The resulting vector was linearized with SpeI, purified and used for pronuclear injections to generate transgenic mice on the FVB/N background as described. 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. 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 microscope (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. 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.

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. 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 and transduced with adeno-viruses expressing HA-tagged β₁-AR or FLAG-tagged β₂-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/aceton 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. Briefly, cell mem- branes were incubated for 1 h at 30°C with 60-100 pmol/L [125I]-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. 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 sucrose, 150 NaCl, 1 EGTA, 2 CaCl₂, 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 calasequestrin (Thermo Scientific) and a monoclonal GAPDH antibody (HyTest). To analyze PKA substrate phosphorylation, 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. 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 Na₂CO₃ 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 β₁-AR (sc-568) and β₂-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.

**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 KH₂PO₄, 1.25 MgSO₄, 24
NaHCO₃, 1.25 CaCl₂ and 11.1 glucose; constantly oxygenated with 95 % O₂ and 5 % CO₂) 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 infarction (MI) model** involving left anterior descending coronary artery ligation to induce chronic heart failure in mice was performed exactly as previously described. Operated animals were subjected to echocardiography and subsequent cardiomyocyte isolation 12 weeks post-MI.
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. (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 β2-AR-cAMP signals. Representative single-cell FRET recordings with the cytosolic (Epac1-camps) and the membrane (pmEpac1) sensors. (A) Selective stimulation of β1-AR with 100 nmol/L of ISO plus 50 nmol/L of the β2-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 β2-AR stimulation with 100 nmol/L ISO plus 100 nmol/L of the β1-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 β1- and β2-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 β1-AR responses are not significant. (D) Comparison of sensitivities between the two sensors using the previously established protocol. Cells were pretreated with 100 µmol/L MDL-12330A for 10 min to inhibit the basal adenyl 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, 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. 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 β1-AR-stimulated cAMP (β1-AR-cAMP) responses were indistinguishable, suggesting that global ISO responses are predominantly mediated by β1-AR. Therefore, we used ISO in all subsequent experiments to stimulate this global β1-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 $\beta_1/\beta_2$-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 (125I-cyanopindolole) competition displacement curves for membranes isolated from TAC and sham myocytes. The first component of the curve represents the $\beta_2$-AR, the second phase – the $\beta_1$-AR fraction. The $\beta_1/\beta_2$-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 β₁-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 described⁵ to induce chronic heart phenotype which was accompanied by ~80% increase in heart-to-body weight ratio, ~60% reduction in fractional 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) β₁- and β₂-ARs were selectively stimulated with 100 nmol/L of ISO plus 50 nmol/L of the β₂-AR antagonist ICI118551 (ICI) and with ISO plus 100 nM of the β₁-AR antagonist CGP20712A (CGP), respectively. D, Quantification of the FRET results shown in A-C demonstrated a pronounced β₁-AR-cAMP desensitization and unchanged β₂-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 β2-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. β1-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. β2-AR was prestimulated with ISO plus CGP. (B, D) Contributions of individual PDEs into the regulation of β1- and β2-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 β2-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-β₁-AR or FLAG-β₂-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 β₁-AR, β₂-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 \(\beta_2\)-AR-stimulated cardiomyocyte contractility in hypertrophied cells. (A and B) Measurements of sarcomere shortening in healthy vs. diseased cells selectively stimulated with \(\beta_2\)-AR ligands (ISO+CGP) and subsequently treated with ANP (100 nmol/L), cilostamide (Cilo, 10 \(\mu\)mol/L) and IBMX (100 \(\mu\)mol/L). Positive effects of ANP and cilostamide on the contractile force after \(\beta_2\)-AR stimulation are abolished in diseased cells. (C and D) BAY60-7550 (BAY, 100 nmol/L) after \(\beta_2\)-AR stimulation leads to an increase in contractility only in TAC cardiomyocytes. Data analysis is shown in Figure 4E. As shown previously, \(\beta_2\)-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 \(\beta_2\)-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 β2-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


