The second messengers cAMP and cGMP play a key role in heart function both in normal and pathological conditions. cAMP mediates the chronotropic, inotropic and lusitropic effects of catecholamines and is involved in the pathogenesis of a number of conditions including hypertrophy, arrhythmia and heart failure (HF). cGMP mediates the cardiac effects of nitric oxide (NO) and natriuretic peptides and has been involved both in negative metabolic and inotropic effects as well as in cardioprotective mechanisms. Depending on the underlying disease, treatment for acute HF relies on inotropes, acting on cAMP signaling, and vasodilators, acting on cGMP signaling. However, lack of a full understanding of the complexity of these pathways limits therapeutic effectiveness and HF mortality remains high.

The reduced intracellular content of cAMP and PKA substrate phosphorylation that normally associates with HF is thought to contribute to the pathophysiology of the disease. However therapy aimed at increasing cAMP levels, although effective in increasing myocardial contractility in the short term, results in increased mortality in the long term and chronic treatment with β-adrenergic receptor antagonists, which act to decrease cAMP generation, results in improved survival. To explain this apparent paradox the view is emerging that cAMP signaling is compartmentalized and spatial control of signal propagation is paramount for specificity of signaling. Thus, depending on their location, cAMP signals may have different functional effects and changes in the phosphorylation of individual PKA substrates may be either beneficial or harmful, depending on the specific target involved.

cAMP is generated by adenylyl cyclases (ACs) on activation of G protein–coupled receptors (GPCRs) in response to a variety of hormones and neurotransmitters. Its main effector is protein kinase (PK)A, a holotetrameric enzyme composed...
of a dimer of regulatory (R) and 2 catalytic (C) subunits. Cardiac myocytes express 2 isoforms of PKA, PKA-RI and PKA-RII, which localize to different subcellular compartments. PKA-RII is mainly found in the particulate fraction of myocyte lysates and PKA-RI, although being mainly recovered in the soluble fraction, has recently been shown to be anchored to specific subcellular sites in intact cardiac myocytes, albeit with a lower binding affinity than PKA-RII. Localization of PKA is achieved via binding to A kinase anchoring proteins (AKAPs) of the amino-terminal dimerization/docking (D/D) domain of the R subunit and 9), the activity of which has been shown to play a pivotal role in the spatial control of cyclic nucleotide signals in these cells. Conversely, PDEs also provide a means by which cGMP signals can modulate cAMP signals. For example, by binding to the regulatory GAF-B domain at the amino-terminus of PDE2, cGMP potently activates the cAMP hydrolyzing activity of this enzyme. Through such a regulatory mechanism, stimuli that elevate cGMP may attenuate cAMP signals. Conversely, cGMP acts effectively as a competitive inhibitor of PDE3 cAMP-degrading activity by virtue of the much higher catalytic rate of this enzyme for cAMP than for cGMP and similarly high affinity for both cAMP and cGMP. As a consequence, PDE3 provides a means by which an increase in cGMP may lead to an increase in cAMP. Although interplay between cAMP and cGMP signals through PDEs has been suggested to occur in a number of cell types, the impact of cGMP on local cAMP in ventricular myocytes has not been addressed. Here we investigate whether the β-adrenergic response in the PKA-RI and PKA-RII compartments of rat ventricular myocytes is affected by cGMP signals. Using real-time FRET imaging and targeted fluorescent reporters we find that defined, localized cAMP responses to isoproterenol (ISO) are profoundly modulated by cGMP. Importantly, the effect of cGMP is strikingly different in distinct subcellular compartments and depends on the source of cGMP. We identify PDE2 and PDE3 as the effectors of such modulation and provide evidence that cGMP alters the local activation of PKA isoforms affecting the phosphorylation of downstream targets and myocytes contractility. These novel findings unravel new aspects of cAMP/cGMP cross-talk in the heart and suggest previously unrecognized possibilities for therapeutic intervention.

Methods

A detailed description of the generation of constructs, primary culture preparation, cell transfection, Western blotting, and FRET imaging is reported in the expanded Methods section, available in the Online Data Supplement at http://circres.ahajournals.org.

Results

sGC and pGC Generate Spatially Distinct cGMP Signals

To assess the amplitude of the cGMP signals generated by different stimuli in selected subcellular locales we used FRET imaging and cGMP sensors targeted to the subcellular compartments where PKA-RI and PKA-RII normally reside. For this we modified the cGMP sensor Cygnet-2.128 by fusing the D/D domain of either RIα or RIβ at its amino-terminus,
as previously described. As expected, the resulting sensors RI_cygnet-2.1 and RII_cygnet-2.1 showed a distinct subcellular localization when expressed in neonatal rat ventriculo-
cytes (NRVMs) (Figure 1A and Online Figure I). Activation of sGC with the NO donor 
$S$-nitroso-$N$-acetyl-d,L-
penicillamine (SNAP) (100 $\mu$mol/L) generated a comparable 
rise in cGMP in the PKA-RI and PKA-RII compartments 
(Figure 1B). In contrast, on treatment with the pGC 
activator atrial natriuretic peptide (ANP, 100 nmol/L) a 
significantly larger cGMP signal was detected by 
RII_cygnet-2.1 as compared to RI_cygnet-2.1 (Figure 1B). 
Thus, different local cGMP signals are generated on 
activation of sGC and pGC.

**cGMP Affects the cAMP Response to ISO in a** 
**Compartment-Selective Manner**

Using the targeted cAMP sensors RI_epac or RII_epac we 
have previously shown that in ventriculoocytes PKA-RI and 
PKA-RII isoforms localize to spatially distinct subcellular 
compartments within which cAMP signals are generated 
selectively in response to the activation of different GPCRs. 
Activation of $\beta$-ARs generates a pool of cAMP that preferen-
tially activates PKA-RII over PKA-RI. To investigate 
whether cGMP signals may affect the compartment-selective 
cAMP response to catecholamines we measured the cAMP 
response elicited by ISO in NRVMs expressing either 
RI_epac or RII_epac in the absence or in the presence of 
cGMP elevating stimuli. As shown in Figure 2, and in 
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selective inhibition of PDE5 with sildenafil (Online Results and Online Figure II). A similar effect of SNAP on local cAMP signals was observed in adult rat ventricular myocytes (ARVMs) (Figure 2C).

The SNAP-induced inversion is cGMP-dependent as it was completely abolished by the sGC inhibitor 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10 μmol/L) (Figure 2A and 2B).

Analysis of the localization of RI_epac and RII_epac presented in the Online Results excludes that the difference detected by the 2 probes may be attributable to a prevalent cytosolic signal revealed by RI_epac as compared to a more specific and local signal revealed by RII_epac (see Online Results and Online Figures III and IV). In addition, we found that when cAMP levels were detected using the nontargeted cytosolic cAMP sensor Epac1-camps no effect of SNAP on the cAMP response to ISO was detectable (Online Figure V). Thus, although cGMP signals dramatically affect the local cAMP response to ISO with opposing effects in the PKA-RI and PKA-RII compartments, such an effect is not apparent when simply measuring global cAMP.

cGMP Generated by pGC Selectively Modulates cAMP Levels in the PKA-RII Compartment

We next assessed the effect of activation of pGC on local cAMP signals. As shown in Figure 3, and in sharp contrast to SNAP, exposure to 100 nmol/L ANP reduced the cAMP response to ISO in the PKA-RII compartment without affecting the response in the PKA-RI compartment. As before, no significant change in the ISO-dependent cAMP response was detected on ANP treatment when the nontargeted cAMP sensor Epac1-camps was used (Online Figure V). Collectively, these observations show that cGMP affects the cAMP response to ISO, that such an effect is compartment-specific and that this selectivity is attributable to the mechanism.
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Figure 3. ANP reduces the cAMP response to ISO selectively in the PKA-RII compartment. A, Representative kinetics of cAMP changes detected by the targeted cAMP reporter RI_epac or RII_epac in NRVMs on stimulation with 10 nmol/L ISO and 100 μmol/L IBMX. Where indicated, cells were pretreated for 10 minutes with 100 nmol/L ANP. B, Summary of all the experiments performed as in A. ***P<0.001 (n≥15).

through which (and hence the compartment in which) cGMP content is raised.

**PDE2 and PDE3 Mediate the cGMP-Dependent Modulation of cAMP**

cGMP can affect cAMP-mediated signaling by modulating the cAMP-hydrolytic activity of myocardial PDEs: cGMP inhibits the cAMP-hydrolytic activity of PDE3 but stimulates the cAMP-hydrolytic activity of PDE2.22 If the PDE3 and PDE2 activity is exerted in the PKA-RI and PKA-RII compartments, respectively, the stimulatory effect of cGMP on cAMP content in the PKA-RI compartment and the inhibitory effect on cAMP content in the PKA-RII compartment could be explained through this mechanism. To test this notion, NRVMs expressing either RI_epac or RII_epac were treated with 10 nmol/L ISO in the presence of 100 μmol/L SNAP and of selective pharmacological inhibitors of either PDE2 or PDE3. As shown in Figure 4A, the PDE2-specific inhibitor BAY 60-7550 (BAY, 10 μmol/L) significantly reduced the effect of SNAP on the ISO-induced cAMP response in the PKA-RI compartment, without affecting the cAMP response in the PKA-RII compartment. These findings support a mechanism in which PDE2 activity is preferentially coupled to the PKA-RII compartment, such that activation of PDE2 by cGMP leads to a selective increase of cAMP content in this compartment.

Figure 4. Effect of selective PDE2 and PDE3 inhibition. A, cAMP changes induced by ISO in NRVMs expressing the targeted cAMP sensor RI_epac or RII_epac. Where indicated, myocytes were pretreated for 10 minutes with 100 μmol/L SNAP, 10 μmol/L BAY 60-7550, or 10 μmol/L cilostamide (n≥7). B, cAMP changes induced by ISO in NRVMs expressing RI_epac or RII_epac. Where indicated, myocytes were pretreated for 10 minutes with 100 nmol/L ANP, 10 μmol/L BAY 60-7550, or 10 μmol/L cilostamide (n≥7). *P<0.01, **P<0.001, ***P<<0.001.

Selective inhibition of PDE3 with cilostamide (CILO, 10 μmol/L) did not affect the SNAP-induced inversion of the cAMP gradient in response to ISO in either compartments (Figure 4A). This finding is in keeping with the hypothesis that PDE3 activity is preferentially coupled to the PKA-RI compartment, such that inhibition of PDE3 by cGMP leads to a selective increase in cAMP content in this locale. In agreement with the notion that, in shaping the cAMP response to ISO, PDE2 and PDE3 exert a predominant activity in the RII and RI compartments respectively, selective inhibition of PDE2 with BAY generates a larger cAMP response to ISO in the PKA-RII compartment whereas inhibition of PDE3 with CILO generates a larger cAMP response in the PKA-RI compartment (Online Figure VI, B and C).

In another set of experiments (Figure 4B) we assessed how selective inhibition of PDE2 and PDE3 affects the ANP-mediated modulation of the cAMP response to ISO. Pretreatment of NRVMs with BAY (10 μmol/L) completely abolished the effect of ANP in the PKA-RI compartment without affecting the cAMP response in the PKA-RII compartment. Inhibition of PDE3 with 10 μmol/L CILO had no effect on the cAMP response in either compartment. The above data confirm that the effect of ANP on the cAMP response to ISO is dependent on a PDE2 hydrolytic activity that is confined to the PKA-RII compartment.
A Localized Pool of PDE2 Is Required for the Compartment-Specific Effects of cGMP on the cAMP Signals

The above data suggest that the activity of spatially confined PDEs is critical for the effects of cGMP on cAMP. To test this hypothesis we generated a catalytically inactive mutant of PDE2A (dnPDE2A) by introducing mutations at positions 685 and 796. These mutations are located in the catalytic site of the enzyme and completely abolish the ability of the enzyme to degrade cAMP (Online Figure VII). When overexpressed in NRVMs the catalytically dead dnPDE2A will displace the cognate endogenous active PDE2A enzymes from specific signaling complexes to which it is sequestered. Thus, contrary to pharmacological inhibition with BAY, overexpression of dnPDE2A is expected to displace the endogenous, cGMP-sensitive enzyme from its intracellular anchor sites replacing it with a cGMP-insensitive version of the enzyme. As shown in Figure 5A, overexpression of mRFP-tagged PDE2AD485A completely counteracts the effects of SNAP on the cAMP response to ISO in the PKA-RI compartment. Similarly, the expression of mRFP-tagged PDE2AD485A completely abolishes the effect of ANP on cAMP levels in PKA-RII compartment (Figure 5B). Taken together, these data provide strong evidence that PDE2 is instrumental in effecting the cGMP-mediated control on cAMP levels and that sequestered PDE2 localization in the PKA-RII compartment is required for achieving the observed compartment-selective modulation of cAMP signals by cGMP.

cGMP Signals Affect Local, Isoform-Specific PKA Activation, and the Phosphorylation of Downstream Targets

Our aim here was to assess whether the effect of cGMP signals on the local cAMP response to ISO would also impact on the downstream activation of individual PKA isoforms. To address this, PKA isoform-selective activity was measured using the FRET sensors RI_AKAR3 or RII_AKAR3. These are genetically modified versions of the FRET-based PKA activity reporter AKAR3 in which the D/D domain of either R1α or R1β was fused at the amino-terminus of the sensor to achieve selective targeting (Figure 6A and Online Figure IX). In agreement with our previous findings, and in keeping with our present results, Figure 6B shows that NRVMs expressing either RI_AKAR3 or RII_AKAR3 and challenged with 0.5 nmol/L ISO have a significantly higher PKA-RI activity compared to PKA-RII. In striking contrast, when cells were pretreated with 100 μmol/L SNAP, ISO selectively increased the activity of PKA-RI over PKA-RII. The effect of ISO on local PKA activity was completely abolished when the sGC was inhibited with 10 μmol/L ODQ (Figure 6B). In keeping with these...
findings, SNAP treatment also significantly reduced the phosphorylation level of the PKA-RII selective targets TnI and PLB (Online Data Supplement and Online Figure X). Similarly, treatment with 100 nmol/L ANP selectively reduced the ISO-induced phosphorylation activity of PKA-RII, with no effect on the activity of PKA-RI (Figure 6C) and reduced the phosphorylation level of TnI and PLB (Online Data Supplement and Online Figure XI). In agreement with these findings, selective pharmacological inhibition of PDE2 with BAY resulted in an increase in the ISO-induced phosphorylation of TnI and PLB, whereas PDE3 inhibition with CILO showed a smaller and not significant effect (Online Figure XII).

The above data demonstrate that the compartment-selective modulation of cAMP levels by cGMP signals propagates to the downstream effectors of cAMP, PKA-RI and PKA-RII, resulting in a dramatic effect on the selective activation of PKA isoforms and target phosphorylation.

cGMP Activation of PDE2 Affects the Contractile Response to ISO

To assess the physiological relevance of the above findings we measured the effect of raising cGMP levels with SNAP on the ISO-induced contractility response of ARVM. We found that treatment with 100μmol/L SNAP significantly reduced myocytes shortening induced by 10 nmol/L ISO (Figure 7A), an effect that was ablated by selective inhibition of PDE2 with 10μmol/L BAY (Figure 7B).

Discussion

In the present study, we examined the effect of cGMP signals on the cAMP response to ISO in rat ventricular myocytes. We found that, although cGMP signals do not change the overall cellular cAMP response, they dramatically affect the cAMP signals locally. Specifically, we found that an increase in cGMP leads to a significant increase in the cAMP pool that activates PKA-RI and to a significant reduction in the cAMP pool that activates PKA-RII. The specific local effect of cGMP on cAMP levels depends on the mechanism through which cGMP content is raised and requires local PDE2 and PDE3 activities. The effect of cGMP propagates to the downstream activation of the cAMP effectors PKA-RI and PKA-RII, leading to altered phosphorylation of PKA targets and affecting myocytes contractility. Based on these findings, we propose a model for cardiac myocytes in
although low cGMP concentrations (<50 nmol/L) exclusively inhibit PDE3, at higher concentrations (between 200 to 500 nmol/L), cGMP also activates PDE2. At high cGMP concentrations, therefore, a possible inhibitory effect of cGMP on PDE3 activity in the PKA-RII compartment may be masked by a prevalent activating effect on PDE2 in this compartment.

The functional consequences of raising cGMP content by activation of pGC and or sGC are different. One possible explanation for this is that cGMP levels increase in specific subcellular compartments depending on the stimulus, thus regulating different phosphodiesterases. A previous study assessing cGMP in the sub–plasma membrane compartment of rat ventriculocytes showed that the particulate cGMP pool is readily accessible at the plasma membrane, whereas the soluble pool is not. Our study, which extends the analysis to compartments localized deep inside the myocytes, confirms that the diffusion of cGMP within the cell is spatially regulated, and the nature of the cGMP-raising stimulus determines which compartment is affected: NO donors, which activate sGC, affect both the PKA-RI and PKA-RII compartments, whereas ANP, which activates pGC, generates a rise in cGMP that is restricted to the PKA-RII compartment. It is worth noting that endogenous NO is generated in a temporally and spatially restricted manner via activation of different NO synthase (NOS) isoforms with distinct subcellular localization, activation mechanisms and regulation, with a potential to generate local cGMP signals that are more complex than those revealed by treatment with exogenous NO donors.

The modulatory effects of NO on cardiac myocytes function are complex and still controversial. For example, NO exerts a positive lusitropic effect that has been attributed to cGMP/PKG-mediated phosphorylation of TnI. On the other hand, NO appears to have mainly negative inotropic and chronotropic effects. NO has been shown to regulate contractility in a bimodal fashion, both in basal conditions and under β-adrenergic drive, with low concentrations of NO exerting a positive effect and high concentrations of NO exerting a negative effect. The specific molecular mechanisms underlying these opposing functions remain unclear, and a variety of possibilities have been implicated, mainly involving PKG-dependent mechanisms or protein nitrosylation. Here we confirm that treatment with high concentration (100 μmol/L) SNAP results in reduced myocytes contractility and our findings indicate that the effects of NO on contractility may be due, at least in part, to the PDE2-mediated modulation of local cAMP levels.

In most studies, the effects of ANP on contractility have been negative, both in vitro and in vivo, although these observations are not universal. The effects of natriuretic peptides on cardiac contractility are important as these peptides become available for clinical use to treat HF. Our results show that ANP, via cGMP-mediated activation of a spatially confined PDE2, significantly reduces cAMP levels in the PKA-RII compartment, a domain that is involved in the control of excitation-contraction coupling, suggesting that cGMP-mediated activation of PDE2 may contribute to the negative inotropy associated with ANP.

Figure 7. Functional effects of cAMP/cGMP interplay. A, Example tracing showing the fractional shortening recorded in isolated ventriculocytes obtained from adult hearts in the absence or in the presence of 10 nmol/L ISO in control cells and cells treated with 100 μmol/L SNAP. B, Summary of the results obtained from 3 independent experiments performed as in A. C, Summary of the results obtained in 3 independent experiments in which cells were treated as in A but pretreated with BAY 10 μmol/L (n≥11). *P<0.05; ns indicates not significant.

which, in the presence of catecholamines, PDE2 activity is preferentially coupled to the PKA-RII compartment, whereas PDE3 activity is preferentially coupled to the PKA-RI compartment and in which cGMP-mediated activation of PDE2 and cGMP-mediated inhibition of PDE3 are responsible for the opposing effects of cGMP on local cAMP signals (Figure 8). This model may also be relevant for the in vivo functions of other cell systems, such as smooth muscle cells and endothelial cells, in which PDE2 and PDE3 enzymes are coexpressed.33

Our present findings clearly demonstrate that cGMP-mediated activation of a spatially confined PDE2 activity is responsible for the cGMP-mediated regulation of cAMP levels in the PKA-RII compartment. Consistent with this model, even in the absence of cGMP signals, selective inhibition of PDE2 generates a cAMP response to ISO that is larger in the PKA-RII than in the PKA-RI compartments. With respect to the compartmentalization of PDE3, the situation is more complex. The observation that pharmacological inhibition of PDE3 does not significantly affect the cAMP response in the PKA-RII compartment suggests that the contribution of PDE3 to shaping the cAMP response to ISO in this locale is marginal. It must be noted, however, that
The crosstalk between NO/cGMP and adrenergic/cAMP signaling is clinically important because these 2 pathways are often concomitantly targeted to treat cardiovascular decompensation. cGMP-raising agents are valuable as vasodilators in clinical practice, and there is evidence for direct cardioprotective effects in animal models. As NO-mimetic agents, however, nitrates also influence cardiac contractility by blunting the positive response to adrenergic signaling, an effect that is even more pronounced in failing hearts. Our study reveals that the positive and negative effects of cGMP on cAMP levels occur in spatially distinct compartments, via modulation of the activity of spatially confined PDEs. In particular, cGMP-mediated activation of PDE2 in the PKA-RII compartment leads to reduced myocytes contractility. The physical separation of positive and negative effects of cGMP on cAMP levels presented here offers the previously unrecognized possibility that the negative inotropic effects associated with increases in intracellular cGMP could be prevented. From this perspective, inhibition of PDE2 might limit the negative inotropic effects of cGMP-raising agents, increasing their clinical utility. On the other hand, stimulation of the cAMP-hydrolytic activity of PDE2, by blocking rises in cAMP, may contribute to the cardioprotective actions of cGMP-raising agents. Expression, intracellular localization, and activity of NOS isoforms have been shown to change in the presence of cardiac disease, potentially affecting the cGMP-mediated regulation of local cAMP signals; however, whether such local changes contribute to pathology, or may rather be protective, remains to be determined. Further experiments in disease models and ultimately in human cardiac myocytes will be necessary to distinguish between these 2 possibilities.

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Disclosures
None.

References
Novelty and Significance

What Is Known?

- There is evidence that signaling mediated by the cyclic nucleotides cAMP and cGMP is compartmentalized with spatially distinct pools of these second messengers affecting different cellular functions.
- Phosphodiesterases (PDEs), the enzymes that degrade cAMP and cGMP, play a key role in spatial control of cyclic nucleotide signals.
- cGMP can potentially modulate cAMP levels by activating PDE2 and inhibiting PDE3.

What New Information Does This Article Contribute?

- In neonatal rat ventricular myocytes (NRVMs), cGMP modulates the cAMP response to isoproterenol (ISO) in a compartment-specific manner.
- The functional impact of cGMP signals depends on the source of cGMP (pGC or sGC) and relies on the specific PDEs associated with each compartment (PDE2 or PDE3).
- The cGMP generated by sGC decreases cAMP levels via PDE2 activation and increases cAMP levels via PDE3 inhibition.
- The cGMP generated by ANP-mediated activation of pGC selectively decreases cAMP levels via PDE2 activation.

NO/cGMP and adrenergic/cAMP signaling pathways are common targets in the treatment of cardiovascular decomposition. cGMP can activate PDE2 and inhibit PDE3; therefore, stimuli that elevate cGMP can either attenuate or enhance cAMP signals with potentially very different functional effects. The interplay between cAMP and cGMP signaling pathways in cardiac myocytes has not been fully elucidated and this knowledge is critical to better target therapy. Using real-time imaging of intact myocytes, we investigated the impact of cGMP signals on the cAMP response to ISO. We found that, depending on the cyclase that generates it, cGMP differently modulates the local cAMP response in distinct subcellular compartments, affecting protein phosphorylation and myocytes contractility. By acting via differently localized PDEs, cGMP can exert opposing effects on local cAMP levels, via activation of PDE2 in one compartment and inhibition of PDE3 in another compartment. The physical separation of positive and negative modulation of cAMP levels by cGMP revealed in this study offers the previously unrecognized possibility of selectively modulating local cAMP signals to obtain more specific functional effects and, to reduce undesired effects. For example, inhibition of PDE2 might limit the negative inotropic effects of cGMP-raising agents; thereby, increasing their clinical utility.
cGMP Signals Modulate cAMP Levels in a Compartment-Specific Manner to Regulate Catecholamine-Dependent Signaling in Cardiac Myocytes

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SUPPLEMENTAL MATERIAL

DETAILED METHODS

REAGENTS
Atrial natriuretic peptide, isoproterenol, cilostamide\(^1\) and IBMX were from Sigma-Aldrich. SNAP and ODQ were from Cayman. Bay 60-7550\(^2\) was from Alexis. Sildenafil was a kind gift from Sharron Francis, Vanderbilt University School of Medicine, Nashville, Tennessee. All restriction enzymes and shrimp alkaline phosphatase (SAP) were purchased from New England Biolabs. Taq DNA polymerase and PFU DNA polymerase were from Promega. For ligation reactions, Rapid DNA Ligation Kit, from Roche, was used.

NRVMs CULTURE AND TRANSFECTION
Primary cultured neonatal cardiac ventriculocytes were isolated from 1- to 3-day old Sprague Dawley rats as described in\(^3\). Collagenase was from Roche, Pancreatin from Sigma, Laminin (mouse) from BD Biosciences. Phosphate-Buffered Saline (PBS), DMEM High Glucose, MEM199, Horse Serum, New born Calf Serum, Penicilline/Streptomycine (10,000 units of penicillin (base) and 10,000 μg of streptomycin (base)/ml) and Glutamine were from Invitrogen. Ventriculocytes were transfected with Transfectin Lipid Reagent (BioRAD), following the supplier’s instructions. Imaging experiments were performed 24–48 hours after transfection.

ARVMs CULTURE AND INFECTION
Wistar rats aged 16 weeks were killed by stunning and cervical dislocation. Single cardiomyocytes were isolated as previously described\(^4\). Briefly, hearts were rapidly removed, cannulated and perfused in a Langendorff perfusion mode for 4-5 minutes with a base Krebs solution (120 mmol/L NaCl, 20 mmol/L Hepes, 0.52 mmol/L NaH\(_2\)PO\(_4\), 5.4 mmol/L KCl, 3.5 mmol/L MgCl\(_2\), 20 mmol/L Taurine, 10 mmol/L Creatine, 11.10 mmol/L Glucose) and subsequently digested for approximately 7 minutes with 0.6 mg ml\(^{-1}\) collagenase type I (Worthington) and 0.04 mg ml\(^{-1}\) protease type XIV (Sigma). Isolated cardiomyocytes were resuspended in MEM199 medium supplemented with 5 mmol/L creatine (Sigma), 5 mmol/L taurine (Sigma), 2 mmol/L carnitine (Sigma), 1% Pen/Strep and plated on laminin coated 24 mm coverslips in 6-well plates. After 2 hours undigested tissue and unattached cells were washed off and the remaining cardiomyocytes were infected with adenoviral vectors carrying RI_epac and RII_epac and cultured overnight. Adenoviral transduction was carried out at a multiplicity of infection (MOI) of 1000 virus particles per cell (vp/cell). Imaging experiments were performed 24 hours after infection.

CONSTRUCT GENERATION
Generation of AdV5/CMV/RI_epac and AdV5/CMV/RII_epac
RI_epac and RII_epac were cloned into the pShuttle-CMV vector and transferred by homologous recombination into the pAdEasy-1 vector to generate AdV5/CMV/RI_epac and AdV5/CMV/RII_epac (using AdEasy™ XL Adenoviral Vector System from Agilent Technologies - Stratagene Products).

Generation of RI_cygnet-2.1 and RII_cygnet-2.1
Cygnet-2.1 (pcDNA3.1-Cygnet-2.1) was a kind gift from Dr. Wolfgang Dostmann, Departments of Pharmacology and Molecular Physiology and Biophysics, University of Vermont, Burlington. RI\(\alpha\) (amino acids 1 to 64) and RII\(\beta\) (amino acids 1 to 49) dimerization and docking domains
(D/D) were amplified by polymerase chain reaction and inserted between NheI and Xhol restriction sites in the pcDNA3 cygnet-2.1 construct.

**Generation of RI_AKAR3 and RII_AKAR3**
The construct AKAR3 (pcDNA3 AKAR3) was kindly provided by Jin Zhang, Department of Pharmacology and Molecular Sciences, the Johns Hopkins University School of Medicine, Baltimore. RI_AKAR3 and RII_AKAR3 were generated by subcloning Rα and Rβ D/D in the HindIII site of the multiple cloning site of the original AKAR3 construct.

**Generation of mRFP-tagged dnPDE2A and PDE2A_{D485A}**
The catalytically dead mutant of PDE2A (dnPDE2A) was generated by introducing the D685A and D796A mutations in pcDNA3.1-PDE2A (mouse) by site directed mutagenesis. The PDE2A construct was a kind gift from Joseph Beavo, Department of Pharmacology, University of Washington Medical School, Seattle. The cGMP insensitive PDE2A_{D485A} was generated by introducing the D485A mutation by site directed mutagenesis. The mRFP-tagged versions of dnPDE2A and PDE2A_{D485A} were generated by subcloning dnPDE2A and PDE2A_{D485A} as NheI fragments at the N-terminus of mRFP (pEYFP-C1-mRFP).

**WESTERN BLOTTING**
Cardiac myocytes treated as indicated were washed twice with PBS (phosphate buffered saline) and lysed for 5 minutes with 300 µL PhosphoSafe™ Extraction Reagent (Novagen®) with Complete™ EDTA-free protease inhibitor cocktail tablets (Roche). Protein concentration of lysates was quantified using the Bradford assay (Biorad). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and subsequently transferred to PVDF (Polyvinylidene Difluoride) membranes (Millipore). Antibodies used for detection were: Phospho-Troponin I (cardiac) Ser23/24 (Cell Signaling, 4004), Troponin I (Cell Signaling, 4002), Anti-PLB phospho-Ser16 antibody (Badrilla, A010-12), Anti-Phospholamban Monoclonal Antibody (Thermo scientific, MA3-922). Quantification was performed using the Image J software (http://rsbweb.nih.gov/ij/index.html). Arbitrary phosphorylation units were calculated and results plotted against controls. iNOS arbitrary units were calculated and results plotted against α-tubulin. Plotted results represent the mean of at least three independent experiments with standard error. T-Test was used for statistics.

**Fluorescence Resonance Energy Transfer (FRET) Imaging**
FRET imaging experiments were performed 24-48 hours after transfection or 24 hours after infection of cardiomyocytes. Cells were maintained at room temperature in a Ringer modified saline (NaCl 125 mmol/L, KCl 5 mmol/L, Na₂PO₄ 1 mmol/L, MgSO₄ 1 mmol/L, Hepes 20 mmol/L, Glucose 5.5 mmol/L, CaCl₂ 1 mmol/L, pH 7.4), and imaged on an inverted microscope (Olympus IX81) using a PlanApoN, 60X, NA 1.42 oil immersion objective, 0.17/FN 26.5 (Olympus, UK). The microscope was equipped with an ORCA-AG CCD camera (C4772-80-12AG, Hamamatsu Photonics, UK) and a beam-splitter optical device (Dual-view simultaneous-imaging system, DV2 mag biosystem, Photometrics, ET-04-EM). The FRET filter settings used throughout were: CFP excitation filter ET436/20x, dichroic mirror 455DCLP (all from Chroma Technology) in the microscope filter cube; dichroic mirror 505DCLP, YFP emission filter 545nm, CFP emission filter 480nm (Chroma Technology) in the beam splitter. Images were acquired using CELL®R software (Olympus) and processed using ImageJ (http://rsb.info.nih.gov/ij/). FRET changes were measured as changes in the background-subtracted 480/545 nm fluorescence emission intensity (for RI_Cygnet-2.1, RII_cygnet-2.1, epac1-camps, RI_epac and RII_epac) or 545/480 nm (for RI_AKAR3 and RII_AKAR3) on excitation at 430 nm and
expressed as either \( R/R_0 \), where \( R \) is the ratio at time \( t \) and \( R_0 \) is the ratio at time \( = 0 \) s, or \( \Delta R/R_0 \), where \( \Delta R = R - R_0 \). Values are expressed as the mean ± SEM.

**PHOSPHODIESTERASE ASSAY**

dnPDE2A activity was assayed by a modified Thompson and Appleman 2-step procedure\(^9\) as described in\(^10\).

**CELL FRACTIONATION**

NRVM fractionation was performed by using the subcellular protein fractionation kit (Thermo scientific, 78840) following the suppliers’ instructions. Protein concentration of different fractions was determined using the Bradford assay (Biorad). Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and subsequently transferred to PVDF (Polyvinylidene Difluoride) membranes (Millipore). Antibodies used for detection were: anti-GFP (sc-9996, Santa Cruz), anti-vimentin (ab-584621, Abcam), anti-HSP90 (ab-13495, Abcam), anti Bcl-2 (sc-783, Santa Cruz), anti Histone H4 (39269, Active Motif).

**FRACTIONAL SHORTENING MEASUREMENTS**

Isolated adult rat ventricular cells were placed in a perfusion chamber mounted on the stage of an inverted microscope and superfused at 37°C with Krebs-Henseleit buffer containing 1mM CaCl\(_2\). Mechanical properties of the myocytes were measured using a video-based edge detection system at 100Hz (IonOptix, Milton, MA, USA). Cells were field-stimulated at 1Hz through a pair of platinum wires placed on opposite sides of the chamber. End diastolic and peak systolic length were measured by IonWizard software (IonOptix) and expressed as fractional shortening (% f.s.).

**PDE2 KNOCK DOWN**

Knock down of PDE2A was achieved by using a siGENOME SMARTpool siRNA, a mixture of 4 siRNA targeting the PDE2A gene (125 nM) (M-094898-00-0020, Rat PDE2A, NM_031079, Thermo scientific). NRVMs were transfected using Transfectin Lipid Reagent (BioRAD) following the supplier’s instructions. Control experiments were carried out using siGLO Red transfection indicator (125 nM) (D-001630-02-20, Thermo scientific).

**REAL-TIME RT-PCR**

Total RNA was isolated from cultured rat neonatal cardiac myocytes, by using the TRIzol reagent (Invitrogen, USA). An aliquot of total RNA (2 µg) was reversed transcribed by 1 µ Superscript II (Invitrogen, USA), 1 µ RNAsin, oligo dT primer (0.5 µg), 10 pmol dNTPs 4 µl 5x first strand buffer, 2 µl of 0.1 mol/l dithiothreitol in a final volume of 20 µl. The oligonucleotides used for the detection of cDNA specific for PDE2A were PDE2A2_eXIXfrd: GCCCAGTACCGCAGC and PDE2A2_eXXrev: CGAGGAGTATAGGTGAAGTTGG. Detection of HPRT was performed with the primers F: 5’AGTCCAGCGGCTCTGATTAG 3’ and R: 5’CCATCTCCTTCTGACATCTCG 3’. Real time PCR assay was carried out with LightCycler (Roche Diagnostics, Germany) using QuantiTect SYBR Green PCR kit (Qiagen, USA). cDNA was added to 10 µl QuantiTect SYBR Green Master mix, 10 pmol of each primer in a total volume of 20 µl in water. Cycling conditions were as follow: denaturation 95°C for 15 min, followed by 50 cycles of 95°C for 15s, 55°C for 1min. Data were collected from two independent mRNA preparations and in each experiment samples curves for calibration of data were created using cDNA derived from untreated cardiomyocytes. The relative quantity of each transcript was determined using the comparative Ct method by interpolating the Ct values of the unknown samples to each standard curve. Values were then normalized with respect to HPRT gene. Melting curve analysis was also performed after PCR amplification to confirm that there were no
primer dimers in the PCR products. The identity of the PCR products of all genes was verified by DNA sequencing.

STATISTICS
Data are presented as average ± Standard Error. Non-parametric t-test was used to compare groups of experiments. Number of replicates is indicated in the figure legends. *0.01<p<0.05, **0.001<p<0.01, ***<0.001.
**SUPPLEMENTAL RESULTS**

**Increasing cGMP levels by inhibition of PDE5 affects the cAMP response to ISO in a compartment-selective manner**

The ability of cGMP signals to affect the specific local cAMP response was confirmed in experiments in which NRVMs expressing either RI\_epac or RII\_epac were treated with the PDE5 inhibitor sildenafil (SILD). As shown in Online Figure IIA, B, ISO generated a higher response in the PKA-RI compartment than in the PKA-RII compartment. Inhibition of PDE5 resulted in a significant decrease in the ISO-induced cAMP response in the PKA-RII compartment and in a significant increase in the cAMP response in the PKA-RI compartment. When the level of cGMP generated by 1 \( \mu \)mol/L SILD in the PKA-RI and PKA-RII compartments was assessed in NRVMs expressing either RI\_cygnet-2.1 or RII\_cygnet-2.1, we found that PDE5 inhibition generated comparable levels of cGMP in the two compartments (Online Figure IIC). These results confirm that an increase in cGMP content exerts diametrically opposite effects on the cAMP response to ISO in the PKA-RI and PKA-RII compartments, resulting in an increase in the cAMP level in the PKA-RI compartment and a decrease in cAMP in the PKA-RII compartment.

**Characterization of the distribution of RI-Epac and RII-Epac and its impact on signal specificity**

Further characterization of the probes was performed to exclude any artifacts due a different localization of the two targeted sensors RI\_epac and RII\_epac. To this purpose subcellular fractionation of NRVMs trasfected with RI\_epac or RII\_epac was performed to step-wise extract cytosolic, membrane (plasma, mitochondria and ER-golgi), nuclear soluble, chromatin bound and cytoskeletal proteins. As shown in Online Figure III, RI\_epac and RII\_epac show an overall similar distribution among all fractions, although in a slightly different proportion. RI\_epac distribution is as follows: 34% in the cytosolic fraction, 19% in the membrane fraction, 2% in the nuclear soluble fraction and 45% in the cytoskeletal fraction. RII\_epac distribution is: 42% in the cytosolic fraction, 23% in the membrane fraction, 9% in the nuclear soluble fraction and 26% in the cytoskeletal fraction.

PKAI and PKAII compete for the binding to intracellular AKAPs. In order to establish if overexpression of the targeted FRET sensors, in particular RI\_epac could in principle displace RII\_epac and contaminate the signals generated selectively in the PKA-RI compartment, we analyzed the relationship between probe expression level and FRET change. We randomly selected 100 cells within the pool of all cells analyzed and plotted (Online Figure IV) the fluorescence intensity signal from CFP (as a measure of probe expression level) against the FRET change generated in that specific cell upon ISO stimulation. Regression curves in Online Figure IV show that there is no correlation between expression level of either sensor and the FRET change elicited by ISO stimulation, at least within the range of fluorescence intensities selected for our experiments.

**Effect of cGMP on global cAMP signals**

Global changes in cAMP upon cGMP-raising stimuli were monitored using the cytosolic cAMP sensor Epac1-camps\(^{11}\). We found that increasing cGMP levels by either SNAP (100 \( \mu \)mol/L) or ANP (100 nmol/L) did not significantly affect the global cAMP response to ISO (10 nmol/L). Similarly, no significant change was detected in global cAMP response to ISO when myocytes were treated with ANP or SNAP concomitantly with the PDE2 inhibitor BAY (10 \( \mu \)mol/L) or CILO (10 \( \mu \)mol/L) (Online Figure V).
Genetic knock down of PDE2A abolishes the effect of cGMP on local cAMP levels in the PKA-II compartment.
To further confirm that the ability of SNAP and ANP to reduce the ISO-induced cAMP response in the PKA-RII compartment is dependent on cGMP-mediated activation of PDE2, we genetically ablated PDE2A using a small interference RNA (siRNA) approach. NRVMs transfected with the PDE2A specific siGENOME SMART pool siRNA show a reduction in the level of PDE2A transcript of 85%, as measured by quantitative real-time PCR (Online Figure VIII A). NRVMs expressing RI_epac or RII_epac and in which PDE2A was genetically knocked down the ability of SNAP to reduce the ISO-induced cAMP response in the PKA-RII compartment was completely ablated whereas, in the same conditions, the SNAP-induced reduction in the cAMP response to ISO in the PKA-RI compartment was not affected (Online Figure VIII B). Similarly, genetic ablation of PDE2A in NRVMs completely abolished the ability of ANP to selectively reduce the cAMP response to ISO in the PKA-RII compartment (Online Figure VIII C). In all conditions, no effect was detected when the control oligonucleotide siGLO was used (Online Figure VIII).

The cGMP-dependent modulation of local cAMP pools affects the phosphorylation levels of PKA isoforms targets
The FRET experiments using RI_AKAR3 or RII_AKAR3 show that cGMP signals selectively affect PKA isoforms activation. We next assessed the effect of cGMP on the phosphorylation level of the PKA targets troponin I (TnI) and phospholamban (PLB). We have previously shown that both these targets are selectively phosphorylated by PKA-RII but not PKA-RI in response to ISO stimulation. Western blot analysis of lysates from NRVMs showed a significant reduction in PLB and TnI phosphorylation when cells were treated with ISO in the presence of SNAP (Online Figure X A-B) and in the presence of ANP (Online Figure XIA-B). These data demonstrate that both SNAP and ANP, by reducing the level of cAMP in the PKA-RII compartment, results in reduced PKA-RII activation thus affecting the level of phosphorylation of PKA-RII downstream targets.
We also assessed the effect of selective PDE inhibition on the level of phosphorylation of PLB and TnI induced by 10 nmol/L ISO stimulation. As shown in Online Figure XII, selective inhibition of PDE2 with 10 μmol/L BAY induced an increase in the phosphorylation level of PLB and TnI. Selective inhibition of PDE3 with 10μmol/L CILO generated a smaller, and not significant, increase in phosphorylation of these targets. Selective inhibition of PDE4 with rolipram (ROLI) (10 μmol/L) induced a significant increase in the phosphorylation level of both PLB and TnI.
Online Figure I

(A) Schematic representation of the targeted cGMP sensors RI_cygnet-2.1 and RII_cygnet-2.1. DDRIα, dimerisation and docking domain of regulatory subunit RIα; DDRIIβ, dimerisation and docking domain of regulatory subunit RIIβ; ECFP, enhanced cyan fluorescent protein; cGMP BD, cGMP binding domain; CD, catalytic domain. (B) Localization of targeted cGMP sensors RI_cygnet-2.1 and RII_cygnet-2.1 in NRVMs. Scale bars 10 µm.
Online Figure II

(A) Representative kinetics and summary of experiments (B) showing cAMP changes detected by RI_epac (n=14) or RII_epac (n=7) upon treatment with 10 nmol/L ISO, 1 µmol/L SILD and 100 µmol/L IBMX. (C) Average cGMP changes upon treatment with 1 µmol/L SILD (n=6 and n=5 for RI_cygnet-2.1 and RII_cygnet-2.1 respectively).
Online Figure III

Western blot analysis of RI_epac and RII_epac distribution in subcellular fractions. Numbers on the top indicate different protein fractions as follows:
1 = cytoplasmic
2 = membranes (sarcoplasma, mitochondria and SR-Golgi)
3 = nuclear soluble
4 = chromatin bound
5 = cytoskeletal
Top row shows probe distribution as detected by anti-GFP antibody. The quality of fractionation is shown below using antibodies against HSP90 (for the cytosolic fraction), Bcl-2 (mitochondria), Histone 4 (chromatin bound) and vimentin (cytoskeleton).
Online Figure IV. Analysis of the correlation between probe expression level and FRET response

Each point represents an individual cell out of a sample of 100 cells randomly selected among the total pool of analyzed cells. Squares represent RNVMs expressing the RII_epac sensor and circles represent RNVM expressing RI_Epac sensor. For each cell, the CFP fluorescence intensity (as a measure of probe expression level) is plotted against the FRET change ($\Delta R/R_0\%$) recorded in that specific cell upon ISO stimulation. The equations describing the linear fit reported in the graph are as follows:

RI_epac: $y=7E-04+3.8551 \quad R^2=0.01$

RII_Epac: $y=8E-05+5.5393 \quad R^2=8E-5$
Summary of the experiments showing global cAMP changes detected by the cytosolic cAMP sensor Epac1-camps upon stimulation with ISO 10 nmol/L. Where indicated myocytes were pre-treated for 10 min with 100 µmol/L SNAP, 100 nmol/L ANP, 10 µmol/L BAY 60-7550 or 10 µmol/L CILO. n≥7. *0.01<p<0.05, **0.001<p<0.01, ***<0.001.
Online Figure VI

Summary of experiments showing cAMP changes detected by RI_epac or RII_epac upon acute application of 10 nmol/L ISO, 10 µmol/L BAY and 10 µmol/L CILO. Asterisks indicate that the cAMP response to ISO is significantly higher (p<0.05) in the RII compartment (B) and in the RI compartment (C) in the presence of the PDE2 inhibitor BAY and of the PDE3 inhibitor CILO, respectively. n= 12
Analysis of the wtPDE2A (light grey bars) and dnPDE2A (dark grey bars) cAMP degrading activity expressed as pmol cAMP hydrolyzed * mg protein^{-1} * min^{-1}. 
Online Figure VIII

(A) mRNA levels of PDE2A detected by Real time RT PCR in untreated NRVMs, NRVMs treated with the control oligonucleotide siGLO red or with the PDE2A specific siGENOME SMART pool siRNA (Thermo Scientific).

(B) cAMP changes induced by ISO in NRVMs expressing RI_epac or RII_epac alone, in combination with siGLO or PDE2A siRNA. Where indicated myocytes were incubated for 10 min with 100 µmol/L SNAP or (C) 100 nmol/L ANP. n≥7. *0.01<p<0.05, **0.001<p<0.01, ***<0.001.
Online Figure IX

(A) Schematic representation of the targeted PKA activity reporters RI_AKAR3 and RII_AKAR3. DDRIα, dimerisation and docking domain of regulatory subunit RIα; DDRIIβ, dimerisation and docking domain of regulatory subunit RIIβ; ECFP, enhanced cyan fluorescent protein; FHA1, phosphoamino acid binding domain; SD, substrate domain; venus, CpV E172 (circular permuted version of the YFP named ‘venus’).

(B) Localization of RI_AKAR3 and RII_AKAR3 in NRVMs. Scale bars are 10 µm.
Online Figure X

Western blot analysis of NRVMs lysates treated as indicated and probed for total PLB (A) and total Tnl (B) and relative phospho-proteins. Quantifications are means of at least three independent experiments. Error bars indicate SE.
Online Figure XI

Western blot analysis of NRVMs lysates treated as indicated and probed for total TnI and total PLB, and relative phospho-proteins. Quantifications are means of at least three independent experiments. Error bars indicate SE.
Online Figure XII

Western blot analysis of NRVMs lysates treated as indicated and probed for total TnI and total PLB, and relative phospho-proteins. Quantifications are means of at least three independent experiments. Error bars indicate SE.
SUPPLEMENTAL REFERENCES


