FRET biosensor uncovers cAMP nano-domains at β-adrenergic targets that dictate precise tuning of cardiac contractility

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Compartmentalized cAMP/PKA signalling is now recognized as important for physiology and pathophysiology, yet a detailed understanding of the properties, regulation and function of local cAMP/PKA signals is lacking. Here we present a fluorescence resonance energy transfer (FRET)-based sensor, CUTie, which detects compartmentalized cAMP with unprecedented accuracy. CUTie, targeted to specific multiprotein complexes at discrete plasmalemmal, sarcoplasmic reticular and myofilament sites, reveals differential kinetics and amplitudes of localized cAMP signals. This nanoscopic heterogeneity of cAMP signals is necessary to optimize cardiac contractility upon adrenergic activation. At low adrenergic levels, and those mimicking heart failure, differential local cAMP responses are exacerbated, with near abolition of cAMP signalling at certain locations. This work provides tools and fundamental mechanistic insights into subcellular adrenergic signalling in normal and pathological cardiac function.
Physical and emotional stress, via release of catecholamines, activation of G-protein-coupled receptors (GPCRs) and synthesis of 3',5'-cyclic adenosine monophosphate (cAMP), promotes a series of events, known as the fight-or-flight response, aimed at avoiding potential harm. In the heart this involves activation of β-adrenergic receptors (β-AR) and consequent increases in heart rate (chronotropy), contractile strength (inotropy) and relaxation rate (lusitropy), so that cardiac output can be enhanced dramatically to support the increased oxygen demands of the body. The main effector of cAMP, protein kinase A (PKA), phosphorylates key targets in cardiac myocytes, including L-type Ca$^{2+}$ channels (LTCC) at the plasmalemma, phospholamban (PLB) on the sarcoplasmic reticulum (SR), and troponin I (TPNI) and myosin binding protein C (MyBP-C) on the myofilaments. These proteins participate in the coupling of cell excitation to myocyte contraction, a process that is graded by the concentration of intracellular [Ca$^{2+}$]$_i$ at each beat and by adjusting the myofilament Ca$^{2+}$ sensitivity$^1$.

In addition to the β-AR fight-or-flight response, cAMP also mediates signalling by numerous hormones, neurotransmitters and GPCRs, and PKA can also phosphorylate numerous distinct target proteins within the same cell. As a consequence, the cell can potentially respond to a rise in cAMP with a multitude of different, and sometimes opposing, effects$^2$. Given the plethora of PKA targets within the same cell, the appropriate physiological response to a specific stimulus is achieved via compartmentalization of both GPCRs and the cAMP/PKA signal$^3$. Compartmentalized signalling allows different GPCRs to generate unique spatially restricted cAMP pools$^4$ that in turn activate defined subsets of localized PKA$^5$. Phosphodiesterases (PDEs), the enzymes that degrade cAMP, play a key role in the spatial regulation of cAMP propagation, and contribute to defining boundaries of individual cAMP pools$^6$.2. Selective phosphorylation is often achieved through tethering of PKA adjacent to specific targets via a kinase anchoring proteins (AKAPs)$^8$.

Multiple drugs currently in use for the treatment of various conditions, including cardiac disease, target the cAMP/PKA pathway. Given the compartmentalization of cAMP signals, a detailed understanding of the actual organization, regulation and function of individual cAMP compartments may allow targeting individual (versus overall global cAMP in the bulk cytosol) cAMP pools, to yield greater therapeutic specificity$^9$. However, the size and location of distinct cAMP domains, the amplitude and kinetics of the cAMP signal within each domain, as well as the specific functional role of individual domains remain largely unknown.

Although real-time imaging of [cAMP] using fluorescence resonance energy transfer (FRET)-based reporters has enhanced our understanding of compartmentalized cAMP signalling$^{10-12}$, major drawbacks have been the limited resolution of existing reporters and the difficulty in directly comparing cAMP signals detected at different intracellular sites$^{13}$. We use here a combination of computational techniques to develop a novel cAMP FRET-based sensor that can be targeted to different macromolecular complexes, with equal cAMP sensitivities, to afford direct comparison of cAMP signals at multiple subcellular sites. Using this new tool we investigate local cAMP responses to catecholamine in cardiac myocytes. By targeting this novel sensor to key protein complexes that regulate excitation–contraction coupling (ECC) we find that physiologically relevant cAMP signals operate within the nanometer range. The cAMP signals generated upon β-AR activation show remarkable and unsuspected local heterogeneity, dictated by the activity of PDEs. Such local coordination of the cAMP signal maximizes β-AR-induced increases in contractility, but also mediates opposing effects on the myofilament response to Ca$^{2+}$. Our novel approach provides unprecedented accuracy and fidelity in the detection of local cAMP signalling with direct applicability to other cellular systems. We also offer original insight into the regulation of fundamental mechanisms of cardiac contractility with profound implications for the pathogenesis and treatment of heart disease.

Results

Limitations of targeted Epa1–camps reporters. Our strategy to study local cAMP signalling with high resolution was to target multiple FRET reporters to distinct subcellular sites. We initially fused the widely used cAMP FRET sensor Epa1–camps$^{14}$ to protein components of various multiprotein complexes. This sensor features the FRET pair Yellow Fluorescent Protein–Cyan Fluorescent Protein (YFP-CFP) at the amino- and carboxyl-termini of the cyclic nucleotide binding domain (CNBD) of the protein Epa1 (Fig. 1a). When expressed in CHO cells and challenged with a saturating stimulus (FRSK + IBMX) that should produce similar maximal responses at all sites, the targeted sensors exhibited variable maximal FRET changes. In the extreme case, no detectable FRET change was seen with PDE4A1-Epa1–camps, as previously reported$^{15}$ (Fig. 1b). Thus, these sensors are impractical for comparing cAMP signals at different targeted cellular sites.

Design of a ‘universal’ FRET tag for cAMP. To overcome the limitations of the Epa1–camps chimeras, we sought to design a FRET sensor based on a novel topology that could potentially serve as a ‘universal’ FRET moiety to tag proteins of interest without affecting FRET sensor properties. We investigated moving YFP from the amino terminus of the sensor to minimize potential interference from the targeting domain (TD) target interaction with the FRET module. As the cAMP-sensing moiety we chose the second CNBD of the regulatory subunit type IIβ of PKA (PKA-RIIβ) (Supplementary Fig. 1a). Sequence conservation analysis (Supplementary Fig. 1b) led us to predict that loop 4–5 within this CNBD may be a suitable point to insert the YFP with minimal risk of perturbing the folding of either the CNBD or the YFP (Fig. 1c). We named this novel sensor CUTie (cAMP Universal Tag for imaging experiments) to emphasize its versatility in fusion proteins. Coarse-grained molecular dynamics (MD) simulations of CUTie using the SIRAH force field$^{16}$ predicted that in the presence of cAMP the distance between CFP and YFP is 6.4 nm, resulting in an average FRET change of 19% upon ligand binding (Supplementary Fig. 1c and Supplementary Note 1).

Generation and characterization of CUTie. The construct for expression of CUTie (Fig. 1c) was assembled and the reporter expressed in CHO cells. Figure 1d shows a representative time course of CUTie FRET change upon application of a saturating stimulus. As predicted from MD simulations, and unlike Epa1–camps, where binding of cAMP results in decreased FRET$^{14}$, cAMP binding to CUTie increases FRET (Fig. 1d, inset). The sensitivity range and EC$_{50}$ for cAMP (Fig. 1e) were determined by measuring FRET changes in CHO cells expressing CUTie and in which intracellular [cAMP] was equilibrated with known [cAMP] in patch pipettes (after membrane rupture). Fusion of CUTie to a number of targeting proteins (Fig. 1f) did not significantly alter the kinetics or the maximal amplitude of the FRET change (Fig. 1g), including fusion to PDE4A1, which had abolished FRET when fused to Epa1–camps (Fig. 1b).
Expression of targeted CUTie in cardiac myocytes. When expressed in neonatal rat ventricular myocytes (NRVM) the targeted CUTie reporters showed the expected specific localization (Supplementary Fig. 2a). For further studies here we focused on three key multiprotein complexes involved in ECC: the AKAP18δ/SERCA/PLB complex localized at the SR, regulating Ca^{2+} reuptake; the myofilament-localized troponin complex (TPNI/TPNT/TPNC) that regulates myofilaments Ca^{2+} sensitivity; and AKAP79/β-AR/adenyl cyclase/LTCC complex at the plasmalemma that regulates cAMP synthesis and LTCC Ca^{2+} influx. Figure 2a shows the predicted localization of CUTie fused to AKAP18δ, TPNI and AKAP79. When expressed in adult rat ventricular myocytes (ARVM), correct localization of AKAP79-CUTie, AKAP18δ-CUTie and TPNI-CUTie was confirmed (Fig. 2b). Co-immunoprecipitation of GFP and western blotting analysis confirmed that the targeted sensors are part of the expected macromolecular complex in ARVM (Fig. 2c) as well as in NRVM (Supplementary Fig. 2b). Expression of targeted CUTie does not affect basal or β-AR-stimulated myocyte responses (Fig. 2d and Supplementary...
Fig. 2d,e). Critically, targeting of CUTie chimeras to myocyte subcellular compartments does not affect the kinetics or the maximal FRET change, both at saturating (Fig. 2e) and sub-saturating cAMP concentrations (Supplementary Fig 2c–e). The in-cell cAMP concentration–response curves for AKAP79-CUTie, AKAP188-CUTie and TPNI-CUTie are superimposable (Fig. 2f), confirming equivalent cAMP sensitivity and dynamic range. For AKAP79-CUTie EC_{50} is 7.17 μM and Hill coefficient is 1.23; for AKAP188-CUTie EC_{50} is 7.14 μM and Hill coefficient is 1.04; for TPNI-CUTie EC_{50} is 7.24 μM and Hill coefficient is 1.15. The sensors can therefore be used for direct comparison of cAMP changes at the sites where they localize.

Differential local [cAMP] during β-AR activation. We next tested cAMP responses to β-AR stimulation by isoproterenol (ISO) in ARVM expressing AKAP79-CUTie, AKAP188-CUTie or TPNI-CUTie. Figure 3a–c shows that ISO (5 nM) induced significantly smaller and more delayed cAMP response at TPNI than at AKAP188 or AKAP79. Such heterogeneity in the cAMP...
response was detected over a range of ISO concentrations, including at saturating concentrations (Supplementary Fig. 3). Similar results were obtained in NRVM (Supplementary Fig. 4a–c). The time course of ISO-dependent phosphorylation of TPNI and PLB reflects the different kinetics of the cAMP response (Fig. 3d). Notably, cAMP responses were synchronous and of similar amplitude at the three sites when ISO was applied in the presence of the PDE inhibitor IBMX (Fig. 3e–g and Supplementary Fig. 4d–f) or the cells were treated with IBMX alone (Supplementary Fig. 4d–f). Western blot analysis confirmed a faster and increased phosphorylation of TPNI (and comparable to PLB) upon IBMX treatment (Fig. 3h). Thus PDE activity is most strongly limiting [cAMP] at TPNI versus SR or plasmalemmal sites.

Maximal stimulated inotropy requires cAMP nanodomains. PKA-dependent phosphorylation of LTCC and PLB causes larger amplitudes of Ca\(^{2+}\) transient and contraction, whereas PKA-mediated TPNI phosphorylation reduces myofilament Ca\(^{2+}\) sensitivity and limits contraction amplitude. Both PLB and TPNI phosphorylation contribute to speed up relaxation during β-AR stimulation. We hypothesized that differential regulation of cAMP at these sites may be required to optimally coordinate PKA-dependent phosphorylation to achieve maximal enhancement of contraction and relaxation. To test this hypothesis, we measured fractional shortening of ARVM in response to cAMP generated by ISO (compartimental signalling) or IBMX (homogeneous signalling) and measured simultaneously in individual ARVM the bulk cytosolic cAMP signal (assessed using the cytosolic, untargeted FRET reporter EPAC-SH187 (ref. 22)). We found that application of 5 nM ISO results in significantly larger increase in fractional shortening as compared to 100 μM IBMX (Supplementary Fig. 5a–e). We then used a concentration of ISO (0.3 nM) that elicits a global cAMP response, as detected by EPAC-SH187, similar to that resulting from inhibition of PDEs with 100 μM IBMX (Fig. 4a, top panel inset). However, unlike 100 μM IBMX, which generates a clearly detectable and equal FRET change at the three sites (Supplementary Fig. 6b), 0.3 nM ISO generates a response that is below the detection limit of the targeted sensors (Supplementary Fig. 6a). When we measured simultaneously in individual ARVM the bulk cytosolic cAMP signal and sarcomere shortening on application of 0.3 nM ISO (Fig. 4a) or 100 μM IBMX (Fig. 4b), we found that 0.3 nM ISO increased contractility significantly more than 100 μM IBMX (Fig. 4a–d and Supplementary Fig. 6c). Notably, the blunted inotropic effect of IBMX is not due to a weaker Ca\(^{2+}\) response, because IBMX did not limit the rise in Ca\(^{2+}\) transient amplitude versus ISO (Fig. 4e,f and Supplementary Fig. 6d). Thus, with ISO we observed a greater contractile benefit for the same Ca\(^{2+}\) enhancement. That is consistent with lower local [cAMP] at TPNI, weaker TPNI phosphorylation and higher myofilament Ca\(^{2+}\) sensitivity with ISO.

Mathematical modelling of local cAMP signalling. To gain additional mechanistic insight regarding differential myofilament phosphorylation effects, we used a detailed cardiac myocyte model of Ca\(^{2+}\), ionic currents and contraction, which includes cAMP-PKA-dependent signalling at nine known PKA targets (including three myofilament targets, TPNI, MyBPC and titin). This was obtained by incorporating our recent contractile model\(^3\) into our mouse ventricular myocyte model\(^2^4\), to better mimic experimental Ca\(^{2+}\), contraction and PKA targets in rats (Supplementary Fig. 7a and Supplementary Note 2). In the baseline model, cAMP rises uniformly at all PKA targets upon β-AR stimulation. This corresponds to the experimental case of PDE inhibition with IBMX, whereby cAMP rises similarly at the plasmalemma, SR and TPNI. Figure 5a, left shows that our model agrees qualitatively with experimental data, with Ca\(^{2+}\) transient and contraction amplitudes rising with IBMX (versus control) by 114% and 106%, respectively. To mimic the weak TPNI-CUTie changes with ISO versus IBMX, we decreased the cAMP rise at the myofilaments by 50% (TPNI, Titin and MyBPC). However, this limited the increases in Ca\(^{2+}\) transient and especially in contraction, compared to uniform [cAMP] (to 88% and 72%, respectively), contrary to experiments in Fig. 4 where ISO increased contraction by more than Ca\(^{2+}\) transients. The model allows us to test the effect of attenuating cAMP effects at any individual PKA target\(^2^5\), so we attenuated all three myofilament targets individually and collectively (Supplementary Fig. 7b). The only condition that showed a greater increase in shortening versus Ca\(^{2+}\) transients was when only the TPNI effect was reduced (myofilament Ca\(^{2+}\) desensitization), but the MyBPC and titin effects responded fully (bar graphs in Fig. 5a and Supplementary Fig. 7b). Indeed, in that case we found good agreement between simulation and experiments, whereby ISO versus IBMX increased Ca\(^{2+}\) transients less than contraction (88 versus 113%, respectively; Fig. 5a, right). Thus, even within the myofilaments there may be differential regulation of PKA-dependent phosphorylation at TPNI, MyBPC and titin.
To test this prediction experimentally, we measured differences in phosphorylation of TPNI, PLB and MyBPC with ISO versus IBMX (Fig. 5b–d). We also used PKA inhibition (H89, 30 μM) and saturating activation to estimate minimal and maximal phosphorylation levels. Addition of 0.3 nM ISO robustly increased phosphorylation of both MyBPC and PLB, but not TPNI (Fig. 5b–d). In contrast, raising cAMP globally with 100 μM IBMX resulted in strong phosphorylation for all three targets. Together with the data at 5 nM ISO (Fig. 3a,b,e,f), these results further confirm that ISO stimulation generates compartmentalized cAMP signals and differential PKA-mediated phosphorylation, with a blunted cAMP/PKA signal at TPNI compared...
to the other sites. The data strongly support the conclusion that a smaller compartmentalized cAMP signal (as generated by 0.3 nM ISO) is more effective in enhancing inotropy than a significantly larger, homogeneous increase in cAMP (as generated by 100 μM IBMX). The data also confirm the model predictions that limiting TPNI phosphorylation allows greater increase in contraction for a smaller increase in Ca\(^{2+}\) transients. The more gradual recruitment of TPNI phosphorylation at higher levels of β-AR activation...
Figure 5 | Mathematical model of compartmentalized cAMP signalling. (a) Simulated cAMP rise (top), myocyte shortening (middle) and Ca\(^{2+}\) transient (bottom) upon administration of IBMX or ISO at time = 0 s. Inset at the left shows baseline condition, and inset at right shows overlapping baseline condition (grey) and steady-state response to cAMP increase. Bar graph in a shows the ratio of the increase in maximal shortening to increase in Ca\(^{2+}\) transient amplitude induced by IBMX and ISO. (b) Western blotting analysis of cell lysates obtained from ARVM treated with H89 (30 μM), vehicle (CTRL), ISO (0.3 nM), IBMX (100 μM) or 25 μM FRSK + 100 μM IBMX (SAT) for 10 min. Membranes were probed for total and phosphorylated MyBPC (b), TPNI (c) and PLB (d). Graphs show densitometric analysis and present the ratio value of phosphorylated to total protein expressed as percentage after normalization to H89 treatment (taken as zero) and to maximal phosphorylation at saturation (taken as 100%). Values are means ± s.e.m. *P \(\leq\) 0.05, ***P \(\leq\) 0.001. N \(\geq\) 8 independent experiments from at least five biological replicates. One-way ANOVA with Dunnett’s post hoc correction.

Figure 4 | Differential local regulation of cAMP signals is necessary for maximal stimulated inotropy. (a) Representative time course of global cytosolic cAMP change (top) and sarcomere shortening (bottom) recorded simultaneously in the same ARVM expressing the cytosolic FRET reporter EPAC-SH187 on application of 0.3 nM ISO or (b) 100 μM IBMX. Inset at the top of a shows mean FRET change measured in ARVM expressing the cytosolic FRET reporter EPAC-SH187 on application of 0.3 nM ISO or 100 μM IBMX. Bars are means ± s.e.m., no significant difference by unpaired t-test. In a, b cells were paced at 1 Hz. Inserts at the bottom of a, b indicate sarcomere shortening kinetics averaged over the time interval indicated by the black bar. (c) Normalized mean sarcomere shortening kinetics measured at steady state after the application of ISO (0.3 nM) or IBMX (100 μM), as indicated. (d) Effect of 0.3 nM ISO or 100 μM IBMX on sarcomere shortening measured in all experiments as shown in a, b. Shortening is expressed as percent increment over control (before the stimulus) calculated as \((\Delta\text{shortening/shortening}_{\text{control}}) \times 100\), where \(\Delta\text{shortening} = (\text{shortening}_{\text{stimulated}} - \text{shortening}_{\text{control}})\). Unpaired t-test. (e) Averaged normalized Ca\(^{2+}\) transient recorded on application of 0.3 nM ISO or 100 μM IBMX. N \(\geq\) 6. (f) Effect of 0.3 nM ISO or 100 μM IBMX on the amplitude of the Ca\(^{2+}\) transient expressed as percent increase over control (before the stimulus). Unpaired t-test shows no significant difference. Bars are means ± s.e.m. *P \(\leq\) 0.05. For all experiments N \(\geq\) 6 from at least three biological replicates.
Figure 6 | Compartmentalized cAMP signalling in hypertrophic cells. (a) Localization of the targeted CUTie reporters in control (top) and hypertrophic (bottom) NRVM. Scale bar: 10 μm. Average cell size for control myocytes was 351.8 ± 7.3 μm² and for hypertrophic myocytes 674.9 ± 14.8 μm² (N ≥ 216 cells from five biological replicates, P ≤ 0.001). (b) Mean FRET change measured with targeted CUTie reporters in control (C) and hypertrophic (H) NRVM on application of 0.5 nM ISO and (c) 0.5 nM ISO in the presence of 100 μM IBMX. N ≥ 6 from five biological replicates. (d) Mean FRET change measured with targeted CUTie reporters in isolated ARVM from minipump vehicle- (V) and ISO-infused (I) rats, on application of 5 nM ISO and (e) 5 nM ISO in the presence of 100 μM IBMX. N ≥ 8 from at least six biological replicates. Bars indicate means ± s.e.m. Unpaired t-test. (f) Mean FRET change measured with targeted CUTie reporters in isolated ARVM from age-matched control (C) and rats subjected to myocardial infarction (MI) at 16 weeks after coronary artery ligation, on application of 5 nM ISO and (e) saturating stimulus. N ≥ 16 from at least seven biological replicates. In all graphs bars indicate means ± s.e.m. Unpaired t-test applied for comparison between treatment groups, one-way ANOVA and Bonferroni’s post hoc test for comparison among sensors. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

may aid relaxation and assure diastolic relaxation under high sympathetic tone, where higher heart rates limit diastolic time.

TPNI is selectively vulnerable to reduced adrenergic input. Given the reduced [cAMP] rise at TPNI versus PLB on ISO stimulation, we asked whether this subcellular compartment may be particularly vulnerable in conditions such as cardiac hypertrophy and heart failure (HF) where the cAMP response to catecholamine is blunted as a consequence of adrenergic desensitization25. To address this question, we expressed the targeted CUTie sensors in NRVM treated for 48 h with 10 μM norepinephrine, a well-established in vitro model of cardiac hypertrophy (Fig. 6a)26. As shown in Supplementary Fig. 8b the in vitro hypertrophied myocytes exhibit an attenuated global cAMP response to ISO. Detection of [cAMP] with the targeted reporters revealed that whereas the response to 0.5 nM ISO at TPNI is significant in control cells it is almost undetectable in hypertrophic cells (Fig. 6b). Unexpectedly, we found that the cAMP signal at AKAP79 and AKAP188 was not significantly different in control versus hypertrophic cells (Fig. 6b), despite global cAMP being significantly reduced (Supplementary Fig. 8b). Application of 0.5 nM ISO in the presence of 100 μM IBMX abolished any difference between subcellular compartments and between control and hypertrophic cells (Fig. 6c).

We next probed the local cAMP response in hypertrophic myocytes from rats subjected to minipump infusion of ISO for 7 days or failing myocytes from rats subjected to myocardial infarction (MI) upon coronary artery ligation (the characterization of these two in vivo models is summarized in Supplementary Table 1). As shown in Fig. 6d and f, respectively, both ISO infusion and MI result in a dramatic decrease in the cAMP response at TPNI compared to the other sites. Interestingly, in both in vivo models, the response at AKAP188 was also significantly reduced compared to control cells, albeit to a lesser extent (Fig. 6d,f), suggesting that additional mechanisms altering
the regulation of local cAMP signalling at the AKAP18δ/PLB/SERCA complex may come into play at later stages during the myocardial remodelling process.

Using the concentration–response curves for cytosolic (Fig. 1e) and targeted (Fig. 2f) CUTie in combination with an in-cell calibration curve for PKA activation (Supplementary Fig. 9) we can estimate the impact on PKA activity of the cAMP signal generated at the sites studied. As summarized in Table 1, the difference between the cAMP response and the resulting PKA activation at TPNI and at the other compartments is exacerbated by reduced adrenergic input. Importantly, while 0.5 nM ISO applied to healthy NRVM results in significant activation of PKA, it does not generate sufficient cAMP to activate PKA at TPNI in hypertrophic cells. The same stimulus, however, generates comparable activation of PKA at other sites both in healthy and hypertrophic cells.

Discussion
We developed a new FRET-based sensor, CUTie, that affords unprecedented fidelity and accuracy of detection of compartmentalized cAMP by quantitatively reporting and allowing direct comparison of cAMP levels in the environment immediately surrounding macromolecular complexes. This sensor offers a general method for fine mapping of cAMP signals in any cellular system. Although CUTie EC50 for cAMP is higher than for other available cAMP FRET reporters27, its sensitivity range is within physiological intracellular cAMP concentrations and adequate to measure cAMP changes elicited by sub-nanomolar β-adrenoceptor agonist.

We investigate the cardiac response to catecholamines and focus on three complexes that regulate ECC: the AKAP18δ/SERCA/PLB complex at the SR, the AKAP79/β-AR/adenyl cyclase/LTCC complex at the plasmalemma and the troponin complex at the myofilaments. We show that the cAMP signal generated in response to β-AR stimulation differs at these three sites in both amplitude and kinetics. Such heterogeneity does not depend on the distance from the site of cAMP synthesis at the sites in both amplitude and kinetics. Such heterogeneity does not depend on the distance from the site of cAMP synthesis at the sites in both amplitude and kinetics. Such heterogeneity does not depend on the distance from the site of cAMP synthesis at the sites in both amplitude and kinetics. Such heterogeneity does not depend on the distance from the site of cAMP synthesis at the sites in both amplitude and kinetics. 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It is well established that PKA-mediated phosphorylation of the PDE isoform(s) involved in attenuation of cAMP signals at nearly abolished at TPNI, but well-preserved at plasmalemma degradation of local PKA as a consequence of the remodelling upregulation of phosphatase activity or delocalization and abnormality in the failing heart. Numerous studies have reported be noted that this sensor displays identical sensitivity to cAMP-dependent, regulatory subunit type II beta (Prkar2

**Methods**

**Generation of CUTie FRET sensor.** The details of the rational design of the CUTie sensor along with the computational methods applied to define the final protein sequence are provided in the Supplementary material section. A DNA fragment encompassing nucleotides 817–986 of the Rattus norvegicus protein kinase, cAMP-dependent, regulatory subunit type II beta (Prkar2

**Isolation and culture of cardiomyocytes.** ARVM were isolated from 350 to 375 g male Sprague-Dawley rats as described. Briefly, hearts were perfused on a Langendorff apparatus at 37 °C for 4–5 min through the coronary arteries with an oxygenated Ca

**In vivo models and echocardiography.** All procedures were carried out in compliance with the standards for the care and use of animal subjects as stated by the requirements of the UK Home Office (APSA1986 Amendments Regulations 2012) incorporating the EU directive 2010/63/EU. For the in vivo infusion of ISO, male Sprague-Dawley rats (Charles River 310–360 g) were anaesthetized using isoflurane and maintained on a heated pad with monitoring of temperature, pulse oximetry and ECG (MouseMonitor S, Indus Instruments). Mini-osmotic pumps (model 2001, Alzet, USA) containing ISO (3 mg per kg per day) dissolved in 0.5% ascorbic acid (to prevent the formation of toxic oxidation products) were implanted subcutaneously in the subscapular region, under isoflurane anaesthesia. Control animals were implanted with pumps containing 0.05% ascorbic acid. Rats were allowed to recover for 7 days. Echocardiographic data were obtained using an 11.5 MHz phased array 10 S-RS pediatric echo probe. Wall thickness and LV dimensions were obtained from M-Mode measurements taken at the level of the papillary muscles. ISO infusion caused the heart rate of the anesthetized rats to increase from ∼800 b.p.m. to 1100 b.p.m. within 5 min. The respiration rate increased to ∼120 breaths per min and remained elevated for the 7 days. Echocardiographic assessment of cardiac function after 7 days of treatment showed that ISO-treated rats had a significantly elevated heart rate (499 ± 23 versus 355 ± 26 b.p.m.) and ejection fraction (97.0 ± 8.9 versus 88.0 ± 9.0) compared with sham controls. ISO-treated rats were hypersensitive, with a significant increase from 380 b.p.m. to 510 b.p.m. within 5 min. The respiration rate increased to ∼120 breaths per min and remained elevated for the 7 days. ISO infusion increased the heart rate of the anesthetized rats to increase from ∼800 b.p.m. to 1100 b.p.m. within 5 min. The respiration rate increased to ∼120 breaths per min and remained elevated for the 7 days. ISO infusion caused the heart rate of the anesthetized rats to increase from ∼800 b.p.m. to 1100 b.p.m. within 5 min. The respiration rate increased to ∼120 breaths per min and remained elevated for the 7 days. ISO infusion increased the heart rate of the anesthetized rats to increase from ∼800 b.p.m. to 1100 b.p.m. within 5 min. The respiration rate increased to ∼120 breaths per min and remained elevated for the 7 days. ISO infusion caused the heart rate of the anesthetized rats to increase from ∼800 b.p.m. to 1100 b.p.m. within 5 min. The respiration rate increased to ∼120 breaths per min and remained elevated for the 7 days. ISO infusion increased the heart rate of the anesthetized rats to increase from ∼800 b.p.m. to 1100 b.p.m. within 5 min. The respiration rate increased to ∼120 breaths per min and remained elevated for the 7 days. ISO infusion caused the heart rate of the anesthetized rats to increase from ∼800 b.p.m. to 1100 b.p.m. within 5 min. The respiration rate increased to ∼120 breaths per min and remained elevated for the 7 days.
Immunofluorescence staining. Twenty-four hours after infection with AKAP18-CUTie or TPNI-CUTie adenovirus, ARVM were fixed in paraformaldehyde 4% for 15 min, washed with PBS and permeabilized in 0.2% Triton X-100 for 30 min. After blocking with 1% BSA for 1 h, the cells were incubated with goat anti-SERCA2 (c-20) (sc-8094, Santa Cruz Biotechnology, TX, USA, used at 1:100) and anti-Mouse IgG (H + L) Alexa Fluor 554 conjugate or anti-Mouse IgG (H + L) Alexa Fluor 554 conjugate (A20717 and A-21425 Thermo Fisher Scientific, MA, USA, both used at 1:10000) for 60 min at room temperature. ARVM expressing GFP-CUTie were incubated with Wheat Germ Agglutinin Alexa Fluor 594 Conjugate (3 μg/ml) (W11262, Thermo Fisher Scientific, MA, USA) for 10 min at 37°C before fixing the cells with formaldehyde 4%. The fixed cells were rinsed in PBS-0.1% Tween20 and mounted in Ibidi Mounting Medium (Thistle Scientific, UK). Images were acquired with an Inverted Olympus FV1000 confocal microscope.

Pull-down experiments. For pull-down experiments ARVM from one heart or 6 x 10^6 NRVM were plated onto 3 x 10-cm Petri dishes coated with laminin (20 μg/ml) and infected with the indicated adenovirus for 3 h. The cells were washed after 36 h with ADS buffer (106 mM NaCl, 20 mM Heps, 0.8 mM NaH2PO4, 5.3 mM KCl, 0.4 mM MgSO4, 5 mM glucose) and lysed in RIPA buffer (Sigma-Aldrich, UK) supplemented with Complete EDTA-free protease inhibitor cocktail (Roche Diagnostics Limited, UK) for 5 min on ice. The lysates were then collected and plated on a rotating wheel for 20 min at 4°C. Insoluble material was removed by centrifugation at 9,600g for 10 min at 4°C and total protein was quantified by Micro BCA Protein Assay Kit (Pierce Biotechnology Inc., IL, USA). Seven hundred fifty milligrams of proteins were rotated for 2–4 h at 4°C with 30 μl of agarose beads coated with a monoclonal anti-GFP antibody (GFP-Trap_A, tga-10, ChromoTek GmbH, DE). The precipitates were then collected by centrifugation at 16,000g for 3 min and beads were washed five times with ice-cold RIPA buffer. Bound proteins were then eluted in 25 μl of 2 × SDS-loading buffer (Life Technologies) and released from the beads at 95°C for 5 min. Pulled down proteins were separated on SDS-PAGE (5–12% Bis-Tris Plus gel (Thermo Fisher Scientific, MA, USA) and transferred onto nitrocellulose membrane (Amersham Protran 0.45 μm, GE Healthcare Life Sciences, UK). After transfer, the membranes were blocked for 1 h at room temperature in Protein-Free (TBS) Blocking buffer (Thermo Fisher Scientific, MA, USA) and then incubated on a metal chamber and kept in Tyrode solution containing 1.4 mM Ca2+ and 37°C, and paced at 1 Hz using a field stimulator (Myopacer; IonOptix, Milton, MA). At steady state, cells were perfused with ISO 0.3 mM or IBMX 100 μM for about 10 min. The background-subtracted Fura-2 AM signal at 310 nm was recorded and expressed as a ratio of fluorescent light on excitation at 340 and 380 nm (R340/380). The recorded transients were analysed using the IonWizard software (IonOptix, Milton, MA).

Western blot analysis. 2 x 10^6 NRVM were plated onto 6-cm Petri dishes coated with laminin (40 μg/ml)–coated coverslips and cultured overnight. Cells were loaded with 1 μM Fura-2 AM (Molecular Probes, OR, USA) in the dark at room temperature for 15 min and then washed three times. Coverslips were mounted in the chamber in 0.5 ml of Tyrode solution containing 1.4 mM Ca2⁺, continuously perfused with a Tyrode solution containing 1.4 mM Ca2⁺ at 37°C, and a Tyrode solution containing 1.4 mM Ca2⁺ at 37°C, and paced at 1 Hz using a field stimulator (Myopacer; IonOptix, Milton, MA). At steady state, cells were perfused with ISO 0.3 mM or IBMX 100 μM for about 10 min. The background-subtracted Fura-2 AM signal at 310 nm was recorded and expressed as a ratio of fluorescent light on excitation at 340 and 380 nm (R340/380). The recorded transients were analysed using the IonWizard software (IonOptix, Milton, MA).

FRET imaging. FRET imaging experiments were performed 24–48 h after transduction with adenovirus carrying each sensor, as described before23. Cells were maintained at room temperature in a modified Ringer solution (125 mM NaCl, 20 mM Heps, 1 mM Na2PO4, 5 mM KCl, 1 mM MgSO4, 5.5 mM glucose, CaCl2, 2 mM, pH 7.4). ARVM were imaged 18 h after infection and kept at ~35°C in Tyrode solution containing 1.4 mM Ca2⁺. An inverted microscope (Olympus IX71) with a PlanApo 60x, NA 1.4 oil immersion objective was used. The microscope was equipped with a CoolSNAP HQ2 monochrome camera (Photometrics) and a DV2 oil optical beam-splitter (MAG Biosystems, Photometrics). Images were acquired and processed using MetaFluor 7.7.1.3. The FRET sensing domain (Flavoprotein Monomeric Devices), FRET changes were measured as changes in the background-subtracted 480 nm/545 nm fluorescence emission intensity on excitation at 430 nm and expressed as R0/R, where R is the ratio at time t and R0 is the average ratio of the first 8 frames. FRET imaging experiments with ARVM isolated from CR1 rats and age-matched controls were performed using an Orca Flash 4.2 sCMOS camera (Hamamatsu, Japan) attached to an inverted microscope (Nikon TE2000) equipped with a 30 Watt dia-illuminator. The system has an EX436/20 excitation filter combined with DM455 dichroic mirror.

Sarcomere shortening. ARVM with resting sarcomere lengths between 1.50 and 1.80 μm were examined for shortening dynamics. Experiments were performed using an inverted microscope (Olympus IX71) with a PlanApO, 40x, NA 1.3 oil objective. Myocytes were imaged by using the transillumination light path equipped with an additional red filter and recorded by a MyoCam-S CCD video camera (IonOptix Milton, MA). Cells were electrically stimulated at 1 Hz in the presence of 1.4 mM Ca2⁺ at 35 ± 1°C. Amplitude and velocity of sarcomere shortening were calculated and normalized using the video-based sarcomere shortening detection software module of IonWizard (IonOptix, Milton, MA). Parameters from 10 contractions were averaged to minimize beat-to-beat variation and obtain mean values at baseline (control) and for the response to stimulus.

A separate cohort of myocytes were infected with Ad-EPAC-S(118) (ref. 22) and used to measure simultaneously sarcomere shortening and FRET. Cells plated onto laminin-coated coverslips were mounted in a metal chamber and kept in Tyrode solution containing 1.4 mM Ca2⁺ at 35 ± 1°C. FRET and shortening images were separated by an additional beam splitter (600 nm HP dichroic mirror, Cairn, Kent, UK) in the emission path and acquired simultaneously with a CoolSNAP HQ2 and MyoCam, respectively. The two cameras were connected to separate PCs to run the imaging software. FRET was imaged using an inverted microscope (Olympus IX71 with a PlanApO, 60x, NA 1.4 oil immersion objective, 0.70/i360 26.5°) attached to an inverted microscope (Nikon TE2000) equipped with a 30 Watt dia-illuminator. The system has an EX436/20 excitation filter combined with DM455 dichroic mirror.
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Author contributions

M.Z., S.P., D.M.B., N.C.S., Ma.Be., A.K. and E.G. designed experiments. N.C.S., D.M.B., A.K., M.Be., M.Br., M.R.M. and S.M. performed experiments and analysed the data. M.Z. and S.P. wrote the manuscript. C.C., P.W. and J.G. provided in vivo rat models of cardiac disease. M.Z. conceived research question and oversaw entirety of research.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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