

RESEARCH ARTICLE

PDE4B mediates local feedback regulation of β_1 -adrenergic cAMP signaling in a sarcolemmal compartment of cardiac myocytes

 Delphine Mika¹, Wito Richter¹, Ruth E. Westenbroek², William A. Catterall² and Marco Conti^{1,*}

ABSTRACT

Multiple cAMP phosphodiesterase (PDE) isoforms play divergent roles in cardiac homeostasis but the molecular basis for their non-redundant function remains poorly understood. Here, we report a novel role for the PDE4B isoform in β -adrenergic (β AR) signaling in the heart. Genetic ablation of PDE4B disrupted β AR-induced cAMP transients, as measured by FRET sensors, at the sarcolemma but not in the bulk cytosol of cardiomyocytes. This effect was further restricted to a subsarcolemmal compartment because PDE4B regulates β_1 AR-, but not β_2 AR- or PGE2-induced responses. The spatially restricted function of PDE4B was confirmed by its selective effects on PKA-mediated phosphorylation patterns. PDE4B limited the PKA-mediated phosphorylation of key players in excitation–contraction coupling that reside in the sarcolemmal compartment, including L-type Ca^{2+} channels and ryanodine receptors, but not phosphorylation of distal cytosolic proteins. β_1 AR- but not β_2 AR-ligation induced PKA-dependent activation of PDE4B and interruption of this negative feedback with PKA inhibitors increased sarcolemmal cAMP. Thus, PDE4B mediates a crucial PKA-dependent feedback that controls β_1 AR-dependent cAMP signals in a restricted subsarcolemmal domain. Disruption of this feedback augments local cAMP/PKA signals, leading to an increased intracellular Ca^{2+} level and contraction rate.

KEY WORDS: Cyclic nucleotide phosphodiesterase, PDE, cAMP β -adrenergic receptors, Compartmentalization, Cardiac myocytes

INTRODUCTION

The cyclic adenosine monophosphate (cAMP) pathway is crucial for the sympathetic regulation of heart function. Upon β -adrenergic (β AR) stimulation, increased cAMP production activates protein kinase A (PKA), which in turn phosphorylates and regulates key proteins involved in excitation–contraction coupling (ECC), such as sarcolemmal Ca^{2+} channels (increasing the L-type Ca^{2+} current $I_{\text{Ca,L}}$), sarcoplasmic reticulum (SR) Ca^{2+} release channels [modifying ryanodine receptor (RyR2) gating], phospholamban (increasing SR Ca^{2+} pump rate via the sarcoplasmic reticulum Ca^{2+} ATPase SERCA) and contractile proteins (cardiac troponin I and myosin binding protein-C) (Bers, 2002). In parallel, activation of EPACs (exchange proteins directly activated by cAMP) causes branching of the cAMP

signaling pathway to regulate additional components, most notably the Ca^{2+} /calmodulin-dependent protein kinase II (Grimm and Brown, 2010). Given their crucial role in the control of cardiac function, cAMP signals are tightly regulated in their intensity, and temporal and spatial dimensions (Brunton et al., 1979; Mika et al., 2012).

It is now well established that cyclic nucleotide phosphodiesterases (PDEs) play an important role in the regulation of cAMP signaling in the heart. By degrading and inactivating cAMP, PDEs are thought to participate in the compartmentalization of the second messenger and in the generation of microdomains of signaling. Five PDE families, PDE1, PDE2, PDE3, PDE4 and PDE8, are predominantly responsible for cAMP degradation in the heart (Conti and Beavo, 2007; Patrucco et al., 2010). Of these, PDE4 isoforms are crucial in shaping global cAMP signals arising from β_1 AR and β_2 AR stimulation in cardiac myocytes in rodents and in humans (Mika et al., 2013; Molina et al., 2012). The PDE4 family is encoded by four genes (*PDE4A*, *PDE4B*, *PDE4C* and *PDE4D*); however, only *PDE4A*, *PDE4B* and *PDE4D* are expressed in cardiac myocytes (Richter et al., 2005; Richter et al., 2011). These three genes encode multiple isoforms generated through alternative splicing and use of alternative promoters/transcription start sites (Bender and Beavo, 2006; Jin and Conti, 2002).

Most of our understanding on the role of individual PDE4 subtypes in the heart is limited to PDE4D. Ablation of this gene in mice leads to PKA hyperphosphorylation of RyR2 and increased sensitivity to exercise-induced arrhythmias (Lehnart et al., 2005). PDE4D is also localized to other compartments of the cardiomyocyte, including the sarcomeric region, via anchoring to myomegalin (Verde et al., 2001), an A-kinase anchoring protein (AKAP) (Uys et al., 2011), the perinuclear region by an association with mAKAP (also known as AKAP6) (Dodge et al., 2001) and to the sarcolemmal region, where PDE4D3 is recruited to the cardiac K^+ channel via AKAP9 (Terrenoire et al., 2009). PDE4D also associates with the PLB–SERCA complex and regulates the SERCA pump activity in the mouse heart (Beca et al., 2011). PDE4D5, PDE4D8 and PDE4D9 have been shown to associate with β ARs either directly or indirectly by binding to β -arrestin (Baillie et al., 2003; De Arcangelis et al., 2009; Richter et al., 2008; Richter et al., 2013). These complex modes of interaction of β AR subtypes with PDEs shape divergent cAMP signals leading to specific physiological responses. More recently, the PDE4B isoform was identified as a component of the L-type Ca^{2+} channel (LTCC) complex and as the major PDE isoform regulating $I_{\text{Ca,L}}$ current during β AR stimulation (Leroy et al., 2011). In this study, we show that PDE4B is crucial for the regulation of cardiac ECC because its ablation increases the propensity of arrhythmias. However, the molecular mechanisms by which PDE4B affects cAMP and channel function were not addressed.

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Here, we investigated how PDE4B affects cAMP homeostasis in different compartments of cardiomyocytes. Using distinct approaches, our findings demonstrate a restricted function for PDE4B in a submembrane compartment of cardiomyocytes. We identified a local feedback regulation whereby β_1 AR stimulation activates PKA, which in turn phosphorylates and activates PDE4B, thereby desensitizing local cAMP signals. This negative feedback is required for maintaining the local cAMP steady state, which is necessary for fine tuning of ECC. Disruption of this feedback alters cAMP dynamics at the sarcolemma and increases phosphorylation of a subset of substrates involved in ECC, including $Ca_v1.2$ and RyR2, leading to an increase in intracellular Ca^{2+} level and an increased chronotropy.

RESULTS

PDE4B functions in a confined compartment near the sarcolemma of cardiac myocytes

To investigate the function of PDE4B in cardiac myocytes, real-time cAMP measurements using FRET-based probes localized either in the cytosol (Epac2-CYTO) or at the plasma membrane (Epac2-PM) (Fig. 1A) were performed using neonatal cardiomyocytes (NCMs) derived from wild-type and *PDE4B*-knockout (*PDE4BKO*) mice. PDE4B ablation has no effect on

β AR-dependent cAMP accumulation in the cytosol (Fig. 1B), but produces significant changes in the sarcolemmal compartment probed with the Epac2-PM sensor (Fig. 1C). Three parameters of the cAMP transient were altered in *PDE4BKO* compared to wild-type NCMs: maximal cAMP accumulation was increased, the time required for half maximal stimulation was also increased and the rate of decay of cAMP levels from maximum was significantly reduced in the absence of PDE4B (Fig. 1C). The differences in response to isoproterenol (Iso) between wild-type and *PDE4BKO* were abolished by inhibition of PDE4 with rolipram (Fig. 1D). This finding confirms that the cAMP phenotype is indeed caused by PDE4 ablation and the overall cAMP synthetic capacity is not altered in *PDE4BKO* cells.

The above data provide initial evidence that chronic removal of PDE4B affects exclusively a membrane compartment. The possibility that there were compensatory changes in the pattern of PDE4 expression was excluded by monitoring the expression of PDE4A and PDE4D in the *PDE4BKO* myocytes. Consistent with previous observations, no changes in PDE4A or PDE4D expression could be detected (supplementary material Fig. S1). Similarly, no change in rolipram-insensitive PDE activity was detected in the hearts of *PDE4BKO* mice, indicating that no adaptive changes in PDEs other than PDE4 are induced (data not shown). To confirm that PDE4B functions in a restricted

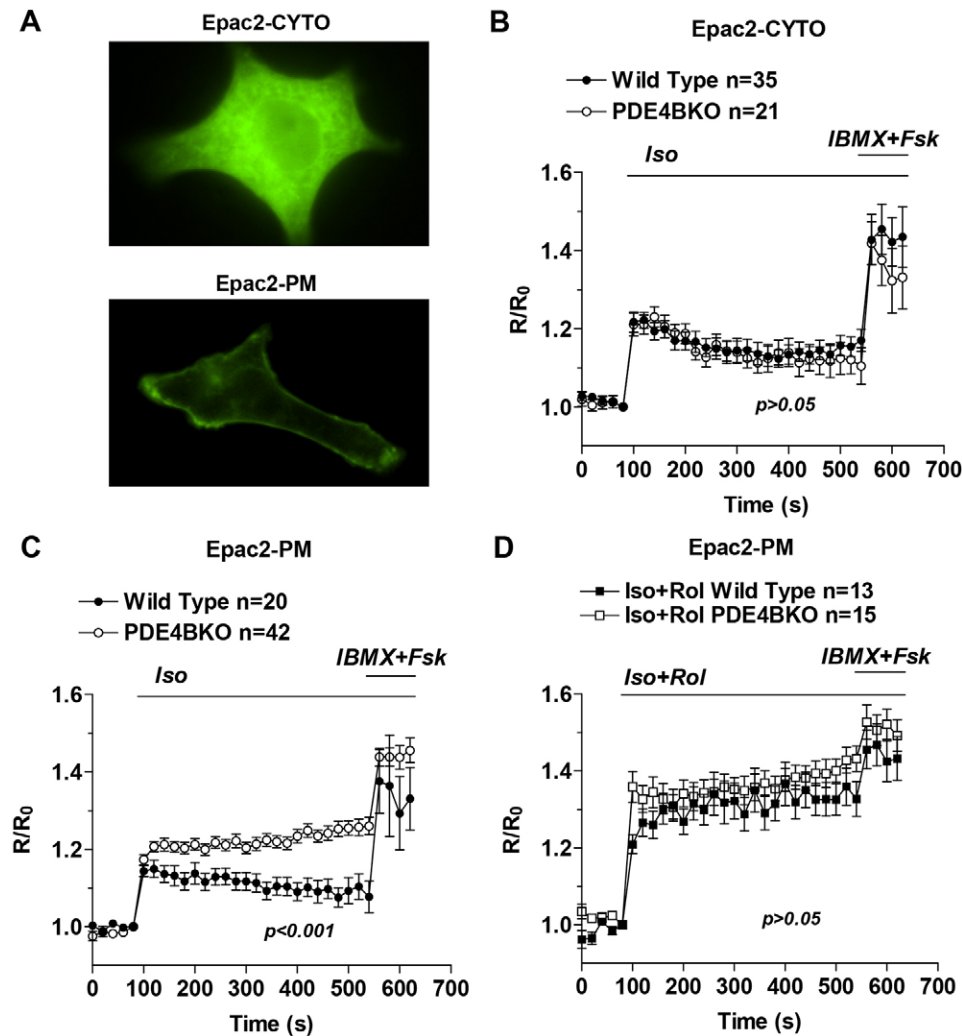


Fig. 1. PDE4B controls subsarcolemmal but not cytosolic cAMP. (A) YFP fluorescence in neonatal cardiac myocytes expressing either Epac2-CYTO (top) or Epac2-PM (bottom). (B–D) Wild-type and *PDE4BKO* myocytes expressing Epac2-CYTO (B) or Epac2-PM (C,D) were treated for the indicated times with Iso (10 nM) alone, or Iso (10 nM) and Rol (10 μ M) followed by IBMX (100 μ M) and Fsk (50 μ M). The average ratio R/R_0 ($R = \text{CFP}/\text{FRET}$) is reported and the number of cells analyzed (n) includes data from at least four experiments. Data were analyzed using a two-way ANOVA. Error bars correspond to the s.e.m. Rol, rolipram; Fsk, forskolin.

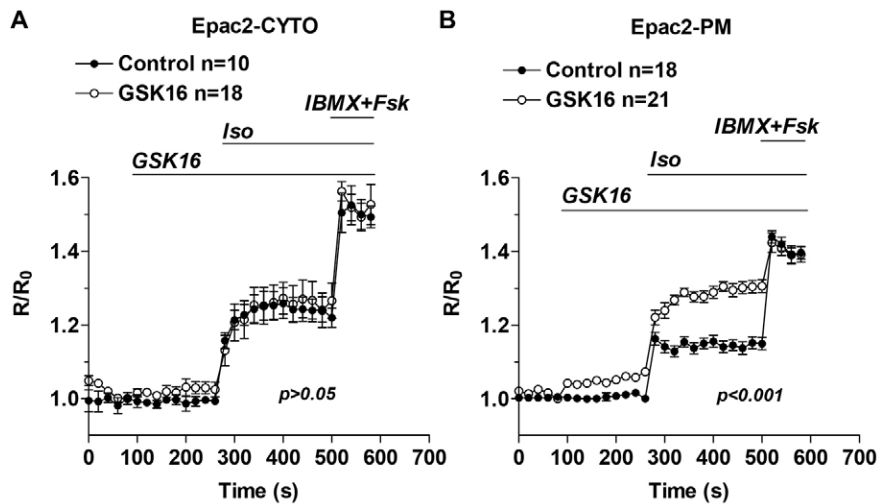


Fig. 2. PDE4B-selective inhibition promotes subsarcolemmal but not cytosolic cAMP accumulation. (A,B) Wild-type myocytes expressing Epac2-CYTO (A) or Epac2-PM (B) were treated for the indicated times with a PDE4B-selective inhibitor (GSK16, 100 nM) and Iso (10 nM) followed by IBMX (100 μ M) and Fsk (50 μ M). The average ratio R/R_0 ($R = \text{CFP}/\text{FRET}$) is reported and the number of cells analyzed (n) includes data from three experiments. Data were analyzed using a two-way ANOVA. Error bars correspond to the s.e.m. Fsk, forskolin.

compartment and that the above data are not the result of long-term compensation for the absence of PDE4B, we used an alternative strategy to probe local PDE4B function. PDE4B-selective compounds have been developed to improve the efficacy and safety of PDE4 inhibition (Donnell et al., 2010; Kranz et al., 2009). We therefore used a PDE4B-selective inhibitor (supplementary material Fig. S2) to probe the consequences of acute PDE4B inhibition on cytosolic and subsarcolemmal cAMP in wild-type NCMs (Fig. 2A,B). This PDE4B-selective inhibitor caused an increase in basal and β AR-stimulated cAMP accumulation in the sarcolemmal compartment (Fig. 2B), which was quantitatively similar to that detected in the PDE4BKO. Moreover the cAMP response to Iso in the cytosol was not affected by the compound, with no statistical difference detected when compared to control (Fig. 2A). Thus, the acute inhibition of PDE4B faithfully recapitulates the PDE4BKO phenotype.

Loss of PDE4B decreases cAMP decay at the plasma membrane

The above measurements at the membrane of NCMs indicate that cAMP concentration in PDE4BKO cells is maintained at a steady state for at least 10 min, suggesting that there is either a local decrease in cAMP hydrolysis or increased cAMP synthesis. To further distinguish between these two possibilities, Iso-mediated stimulation of cAMP synthesis was blocked by using an excess of the β AR antagonist, propranolol (1 μ M) (Fig. 3A,B) to determine cAMP decay. Assuming complete blockade of adenylyl cyclase activation, the rate of cAMP decay should be dependent on PDE activity in the compartment. Under these experimental conditions, the $t_{1/2}$ of cAMP decay was reduced by 50% in the PDE4BKO cells (69.8 ± 8.5 s vs 122.5 ± 23.4 s) confirming a decrease in PDE activity in this compartment (Fig. 3C). The fact that cAMP continues to decrease even in the absence of PDE4B indicates that (1) other PDEs are also involved in cAMP degradation in this compartment, and/or (2) that cAMP diffuses out of this compartment.

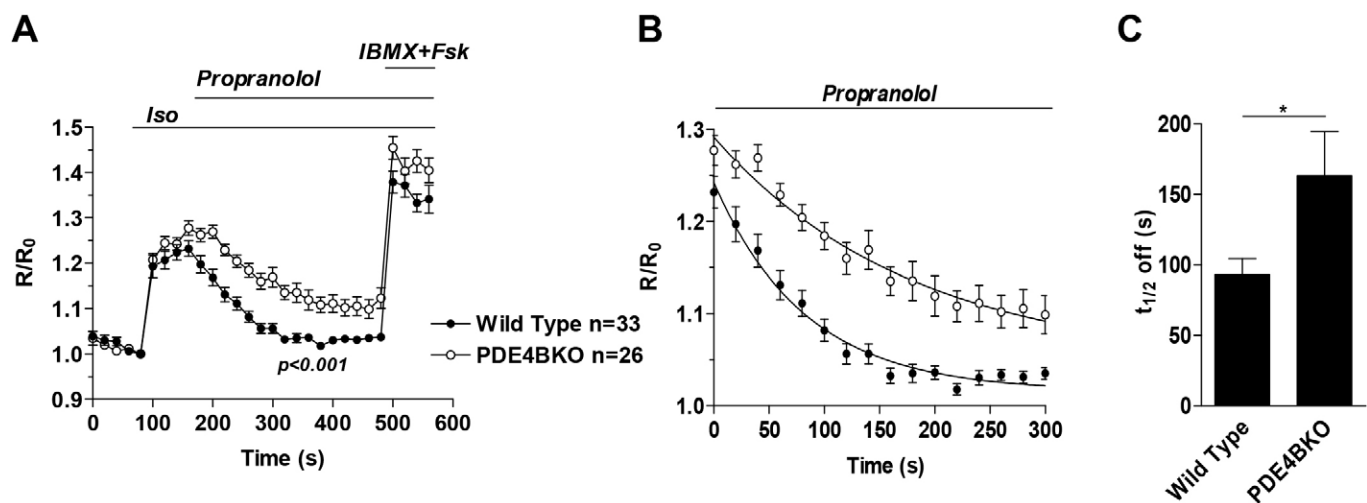


Fig. 3. Rate of cAMP decay at the plasma membrane of wild-type and PDE4BKO myocytes. (A) Wild-type and PDE4BKO myocytes expressing Epac2-PM were stimulated for the indicated times with Iso (10 nM) followed 80 s later by treatment with propranolol (1 μ M). At the end of the incubation IBMX (100 μ M) plus Fsk (50 μ M) was added to measure the maximal response of the cell. The average ratio R/R_0 ($R = \text{CFP}/\text{FRET}$) is reported and the number of cells analyzed (n) includes data from three or more experiments. Data were analyzed using a two-way ANOVA. (B,C) Data were fitted with an exponential decay equation (B) and the average $t_{1/2}$ off was calculated for the two genotypes (C). Data were analyzed using an unpaired Student's t -test; $*P < 0.05$. The number of cells analyzed (n) includes data from at least three experiments. Error bars correspond to the s.e.m. Fsk, forskolin.

PDE4B controls β_1 AR but not β_2 AR or EPR responses in cardiac myocytes

The above findings indicate that there is a localized function of PDE4B in the proximity of the sarcolemma in cardiac myocytes. However, the plasma membrane compartment of these cells is not homogeneous but rather divided into subcompartments, including caveolae and non-caveolar lipid rafts, which contain distinct sets of receptors and signaling proteins (Insel et al., 2005). To further characterize the membrane subcompartment where PDE4B is active, we determined whether PDE4B ablation affects the signaling of different G-protein-coupled receptors (GPCRs). NCMs were preincubated with antagonists to block β_1 AR (CGP-20712A, 1 μ M) or β_2 AR (ICI-118551, 1 μ M) and cells were then stimulated with 10 nM Iso. Under these conditions, PDE4B ablation affects β_1 AR responses (Fig. 4A) but not cAMP signals induced by β_2 AR stimulation (Fig. 4B). Ablation of PDE4B also does not affect the cAMP transients induced by activation of the prostaglandin E receptors (EPRs) by PGE2 (100 nM) (Fig. 4C). Taken together, our data show that PDE4B controls a restricted pool of subsarcolemmal cAMP exclusively associated with β_1 AR activation.

PDE4B controls PKA activity at the sarcolemma but not in other compartments

As PDE4B regulates cAMP signaling in a restricted subsarcolemmal compartment, we wished to determine whether signaling events downstream of cAMP, namely activation of PKA and phosphorylation of its downstream targets, would be similarly restricted. To this end, we measured the phosphorylation level of several PKA substrates located in different subcellular regions of the NCM in wild-type and PDE4BKO cells. PKA phosphorylation of the LTCC subunit $\text{Ca}_v1.2$ as well as the ryanodine receptor (RyR2) and vasodilator-stimulated phosphoprotein (VASP), a component of the membrane actin cytoskeleton, was greatly increased in the

PDE4BKO cardiomyocytes stimulated with Iso (10 nM) (Fig. 5A–C). Conversely, phosphorylation of phospholamban (PLB), which controls the activity of the SERCA pump, was the same in wild-type and PDE4BKO cells (Fig. 5D). Similarly, PKA phosphorylation of two substrates associated with the sarcomere, myosin-binding protein C (MyBP-C) and troponin I (TnI), was not affected by PDE4B ablation (Fig. 5E,F).

In wild-type NCMs, acute and selective inhibition of PDE4B promoted β_1 AR-, but not β_2 -induced PKA-mediated phosphorylation of $\text{Ca}_v1.2$, RyR2 and VASP, whereas PKA-mediated phosphorylation of PLB, MyBP-C and TnI were not altered (supplementary material Fig. S3). These discrete effects on PKA-mediated phosphorylations confirm that PDE4B predominantly functions to control a sequestered cAMP pool in the vicinity of the sarcolemma and that elevated cAMP levels in this compartment following PDE4B ablation do not spill over into other compartments including those containing the myofilament proteins and PLB.

PDE4B mediates a PKA-dependent feedback regulation of β_1 AR-signaling

At least five distinct protein variants, PDE4B1 to PDE4B5, are generated from the *PDE4B* gene. Western blots of NCM extracts with PDE4B-specific antibodies indicate the presence of a single prominent immunoreactive band with an apparent molecular mass of 95 kDa (Fig. 6A). This band corresponds to the PDE4B3 variant (supplementary material Fig. S4). Consistent with this view, immunoprecipitation with specific anti-PDE4B3 antibodies, but not with anti-PDE4B1 antibodies, led to recovery of a 95-kDa band in the immunoprecipitation pellet (supplementary material Fig. S4). This variant is a so-called long PDE4B form that contains a PKA phosphorylation consensus site at its N-terminus, and phosphorylation at this site mediates activation of the recombinant PDE4B protein (MacKenzie et al., 2002). Total extracts from NCMs were subjected to immunoprecipitation with PDE4B or control IgG antibodies. Fig. 6B confirms the

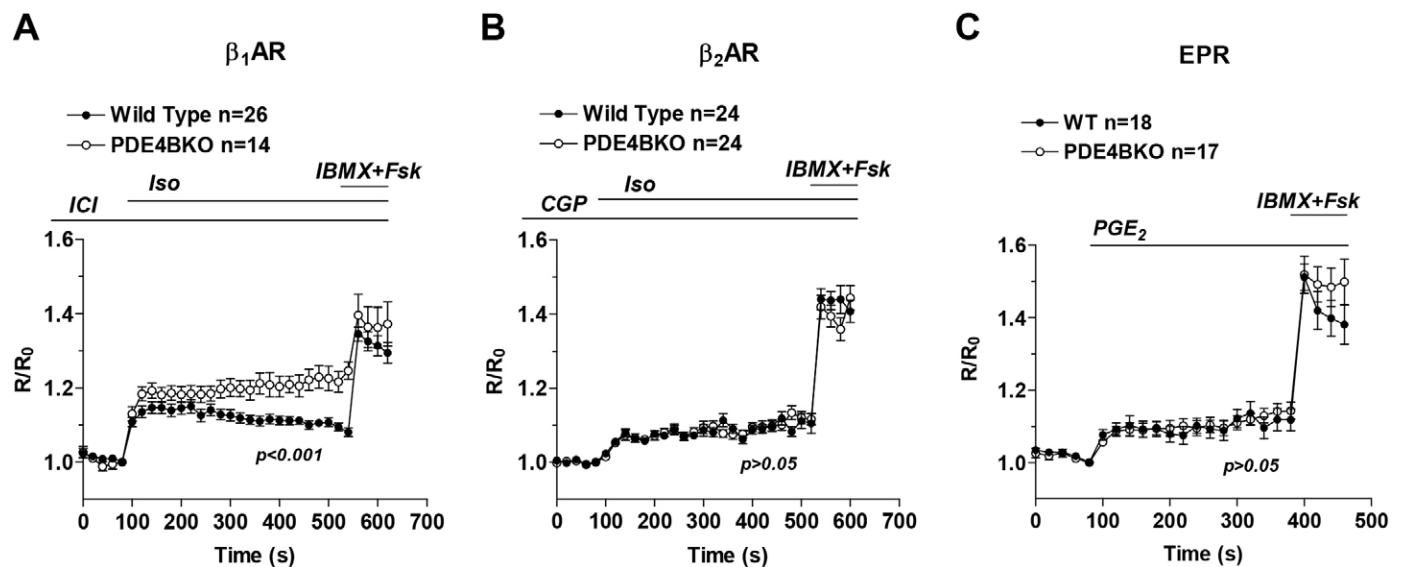


Fig. 4. PDE4B controls β_1 AR, but not β_2 AR or EPR responses in cardiac myocytes. Wild-type and PDE4BKO myocytes expressing Epac2-PM were preincubated with 1 μ M ICI-118551 (ICI, β_2 antagonist) (A) or 1 μ M CGP-20712A (CGP, β_1 antagonist) (B) for 5 min, then stimulated with Iso (10 nM) followed by IBMX (100 μ M) plus Fsk (50 μ M). (C) Wild-type and PDE4BKO myocytes were stimulated with PGE2 (100 nM) followed by IBMX (100 μ M) plus Fsk (50 μ M). The average ratio R/R_0 ($R = \text{CFP}/\text{FRET}$) is reported and the number of cells analyzed (n) includes data from at least four experiments. Data were analyzed using a two-way ANOVA. Error bars represent the s.e.m. Fsk, forskolin.

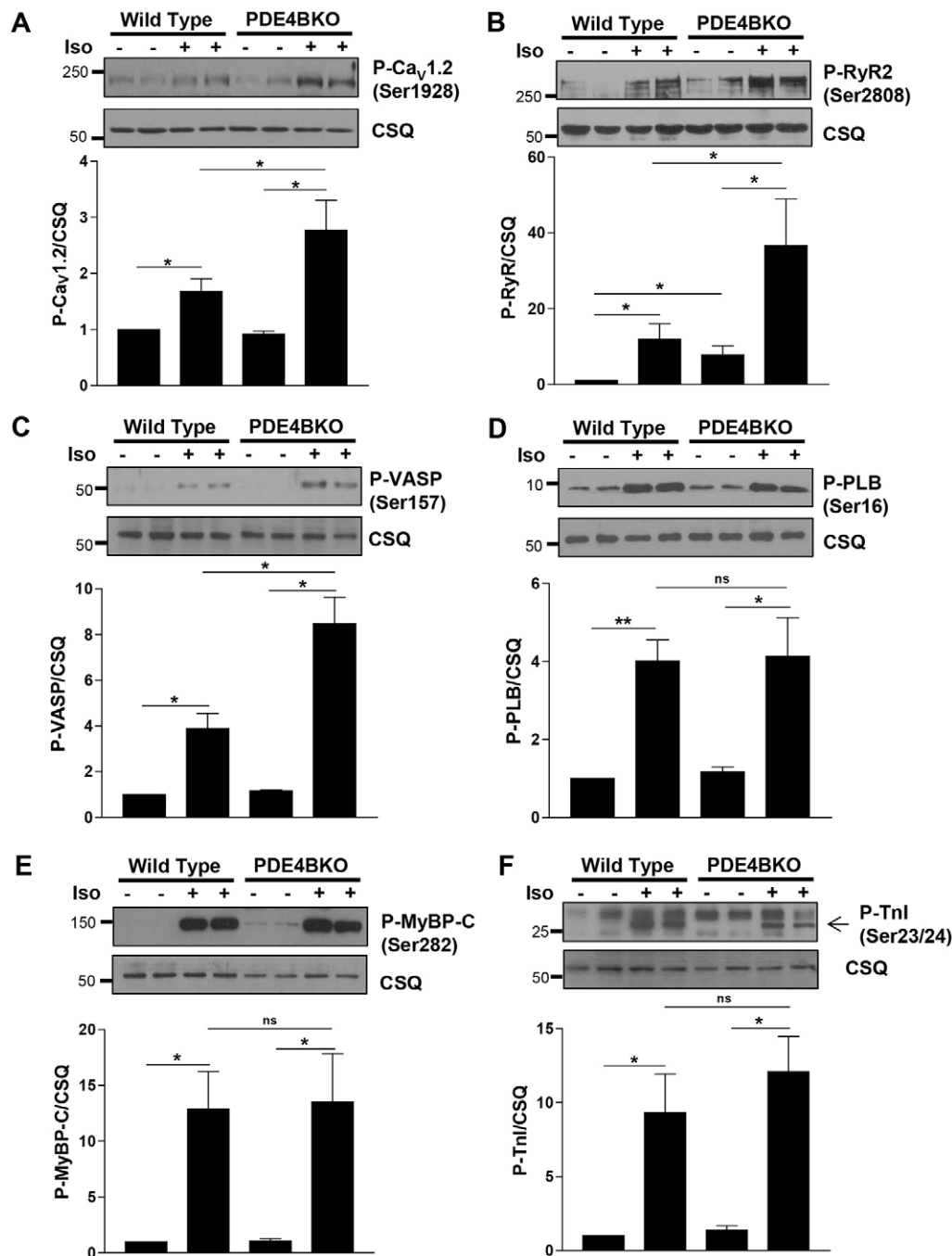


Fig. 5. PDE4B controls PKA activity in a subsarcolemmal compartment, but not global PKA activity. Detergent extracts prepared from wild-type and PDE4BKO myocytes treated for 10 min with or without Iso (10 nM) were probed for phospho-Ca_v1.2 (P-Ca_v1.2, Ser1928) (A), phospho-ryanodin receptor 2 (P-RyR2, Ser2808) (B), phospho-VASP (P-VASP, Ser157) (C), phospho-phospholamban (P-PLB, Ser16) (D), phospho-myosine-binding protein C (P-MyBP-C, Ser282) (E) and phospho-troponin I (P-TnI, Ser23/Ser24) (F). Calsequestrin (CSQ) was used as a loading control. A representative western blot of three performed is included for each substrate. For quantification, the ratios of the immunoblot intensity for the phosphorylated proteins over that for CSQ were normalized to untreated (without Iso) wild-type cells and expressed as mean ± s.e.m. **P*<0.05; ***P*<0.01; ns, not significant (Student's unpaired *t*-test).

specificity of the PDE4B antibody used for immunoprecipitation. No significant immunoprecipitation activity was detected when the experiment was repeated in PDE4BKO myocytes (Fig. 6B). Selective stimulation of β_1 AR, but not stimulation of β_2 AR or EPR, induced a more than 2-fold increase in cardiomyocyte PDE4B activity (Fig. 6C). This β_1 AR-dependent increase in PDE4B activity is reversed when the cells were pretreated with the PKA inhibitor H89 (Fig. 6C). Taken together, these data demonstrate that PDE4B expressed in NCMs is selectively phosphorylated and activated by PKA after stimulation of β_1 AR. Moreover, they provide independent confirmation that PDE4B is involved in the regulation of β_1 AR but not β_2 AR signaling; being activated only when the former receptor is occupied.

PKA-mediated activation of PDE4B is crucial for cAMP signaling at the plasma membrane

To determine the consequences of the PKA-mediated phosphorylation and activation of PDE4B in the control of cAMP accumulation at the plasma membrane, FRET measurements of cAMP in this compartment were performed in the presence or absence of the PKA inhibitor H89 (20 μ M). At this concentration, this inhibitor completely blocked the β_1 AR-dependent activation of the endogenous PDE4B. Inhibition of PKA triggered a significant increase of Iso-stimulated cAMP levels at the plasma membrane of wild-type myocytes (Fig. 7A). Conversely, the effect of H89 was dramatically reduced in PDE4BKO cells (Fig. 7B) suggesting that PDE4B activation is

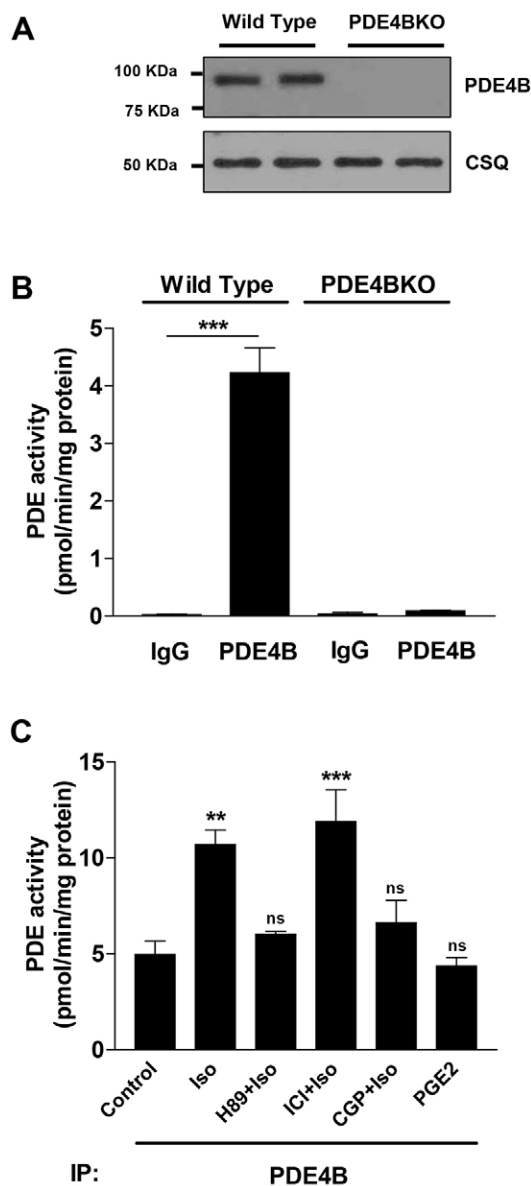


Fig. 6. Phosphorylation and activation of PDE4B in cardiac myocytes. (A) Detergent extracts prepared from wild-type and PDE4BKO NCMs were probed by western blotting with anti-PDE4B antibodies. Calsequestrin (CSQ) was used as a loading control. (B) Total extracts from wild-type and PDE4BKO NCMs were subjected to immunoprecipitation (IP) with anti-PDE4B antibodies or anti-IgG antibodies. Shown is the PDE activity recovered in the immunoprecipitation pellets. (C) Wild-type myocytes were preincubated with mock, PKA inhibitor (H89, 20 μ M, 1 h), ICI-118551 (ICI, β_2 antagonist, 1 μ M, 5 min) or CGP-20712A (CGP, β_1 antagonist, 1 μ M, 5 min), then stimulated with Iso (100 nM) or PGE2 (100 nM) for 10 min. Total extracts were then subjected to immunoprecipitation with anti-PDE4B antibodies and the PDE activity recovered in the immunoprecipitation pellets was measured. For B and C, data represent the mean \pm s.e.m. of experiments performed three times. ** P <0.01; *** P <0.001; ns, not significant (Student's unpaired t -test).

the main mechanism of PKA-mediated feedback regulation of cAMP signaling in this submembrane compartment. In the cytosol, pretreatment with H89 induced comparable increases in cAMP accumulation in wild-type cells (Fig. 7C) and PDE4BKO cells (Fig. 7D), suggesting that a different PDE mediates the PKA effects in the cytosol.

PDE4B ablation increases intracellular Ca^{2+} levels and myocyte contraction rate after β_1 AR stimulation

It is well established that stimulation of β_1 AR signaling increases heart rate (Devic et al., 2001; Xiang et al., 2005) and that this effect is due in part to increased PKA-mediated phosphorylation and activation of $Ca_v1.2$ and RyR2 (Bers, 2008; Shan et al., 2010). The fact that PDE4B controls the PKA-mediated phosphorylation of LTCC and RyR2 prompted us to compare the intracellular Ca^{2+} levels and the contraction rate of wild-type and PDE4BKO NCMs. To this end, we measured Fura-2 ratios and the spontaneous beating rate after β_1 AR stimulation with 10 nM Iso (Fig. 8). We found that Iso-induced intracellular Ca^{2+} levels in PDE4BKO cells are significantly increased compared to wild-type myocytes (Fig. 8A). The contraction rate measured in PDE4BKO cells tends to be higher than in wild-type cells under basal conditions (although not significant) and is significantly increased after Iso stimulation (Fig. 8B). This finding is consistent with the hypothesis that PDE4B ablation evokes an increase in the β_1 AR-stimulated contraction rate of cardiac myocytes, likely via increased PKA-mediated phosphorylation of ECC proteins such as $Ca_v1.2$ and/or RyR2.

DISCUSSION

The data described above demonstrate a local PDE feedback regulation in cardiac myocytes whereby β_1 AR signals activate PDE4B3 via PKA-mediated phosphorylation. PKA-activated PDE4B, in turn, reduces the local steady-state cAMP concentration in a confined subsarcolemmal domain. The functional consequences of interrupting this feedback include increased PKA-mediated phosphorylation of $Ca_v1.2$ and RyR2, which is necessary for fine-tuning of ECC, a finding consistent with the cardiac phenotype of PDE4BKO mice (Leroy et al., 2011). Functional data on intracellular Ca^{2+} levels and contraction rate confirm the altered cAMP/PKA signaling in PDE4BKO myocytes. These conclusions are based on measurements of β_1 AR-induced cAMP accumulation at the plasma membrane of PDE4BKO myocytes, the effects of acute PDE4B-selective inhibition in wild-type myocytes, the β_1 AR-dependent activation of PDE4B and the altered PKA-mediated phosphorylation of some, but not all substrates involved in Ca^{2+} homeostasis and contraction. Further proof for a restricted function of PDE4B is our finding that this enzyme is not regulated by β_2 AR signals and does not control β_2 AR-dependent cAMP accumulation at the plasma membrane. Thus, PDE4B serves a highly specialized and localized function to control β_1 AR-dependent ECC in cardiac myocytes.

The major PDE4B isoform expressed in neonatal and adult mouse cardiac myocytes is a so-called long form that contains a conserved consensus site for PKA phosphorylation (Conti et al., 2003). Here, we show that during β_1 AR stimulation, PKA phosphorylates and activates this PDE4B isoform. Its mobility in SDS/PAGE gels is identical to the PDE4B isoforms identified in the adult mouse, rat and human heart (Abi-Gerges et al., 2009; Richter et al., 2011). Thus, any regulation of this isoform is likely conserved and relevant to human cardiac physiology.

Being a substrate for PKA phosphorylation, activation of different PDE4s by itself might be used to explore the selectivity of receptor signaling as well as define its spatial propagation. We have previously shown that β_1 AR and β_2 AR activate different subsets of PDE4D variants. β_1 AR ligation preferentially activates PDE4D8 localized in the vicinity of the receptor, whereas β_2 AR activates PDE4D9 and PDE4D5 (Richter et al., 2008). This

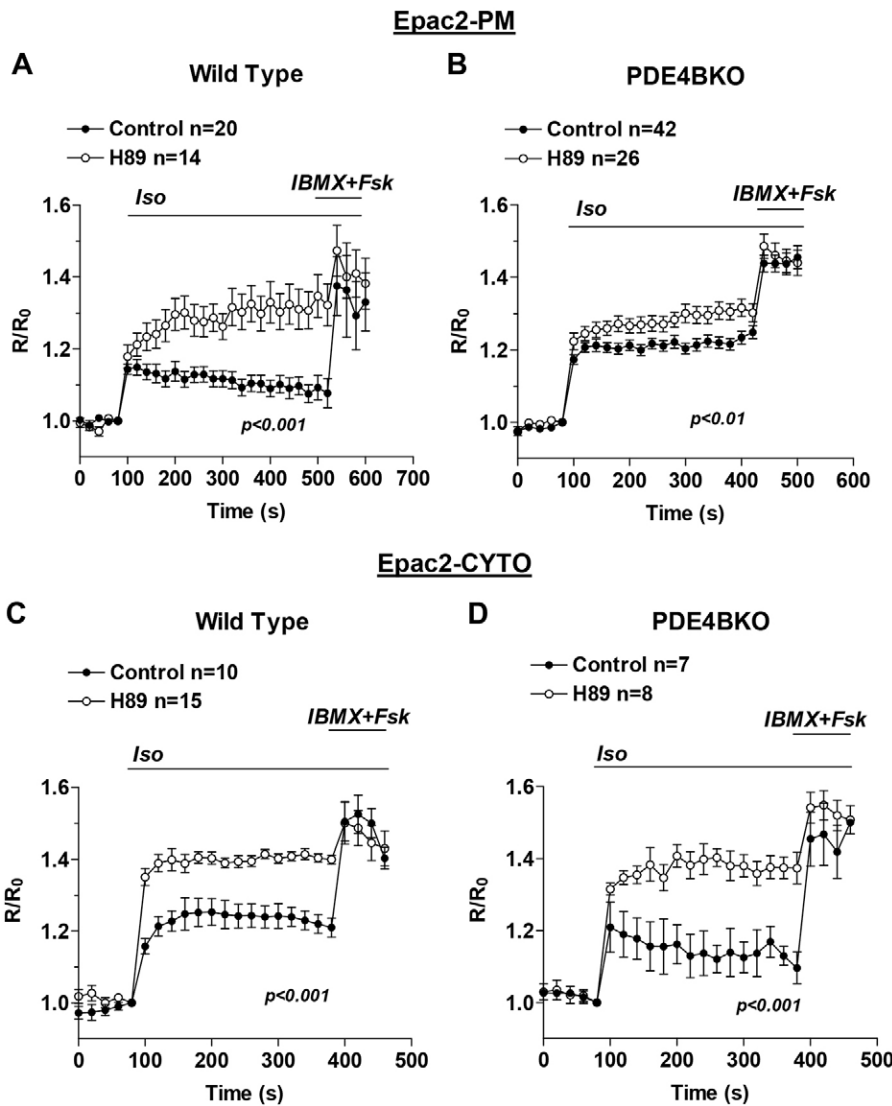


Fig. 7. PDE4B phosphorylation by PKA contributes to the pattern of cAMP accumulation. Wild-type (A,C) and PDE4BKO (B,D) myocytes expressing Epac2-PM (A,B) or Epac2-CYTO (C,D) were pretreated for 1 h with or without H89 (20 μ M) before stimulation for the indicated times with Iso (10 nM) and IBMX (100 μ M) plus Fsk (50 μ M). The average ratio R/R_0 ($R = \text{CFP}/\text{FRET}$) is reported and the number of cells analyzed (n) includes data from four or more experiments. Data were analyzed using a two-way ANOVA. Error bars correspond to the s.e.m. Fsk, forskolin.

concept is reinforced and extended with the demonstration that β_1 AR, but not β_2 AR, signals to activate PDE4B in neonatal cardiac myocytes. Previously, we have shown that PDE4B co-immunoprecipitates with the LTCC in adult mouse ventricular

myocytes suggesting that this isoform is tethered in a macromolecular complex with the channel at the plasma membrane and is located in the proximity of the T-tubules (Leroy et al., 2011). By contrast, PDE4D5 preferentially

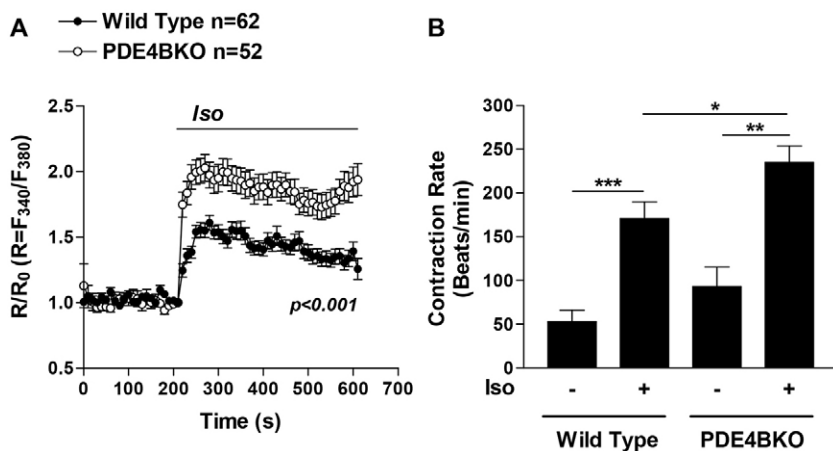


Fig. 8. PDE4B regulates intracellular Ca^{2+} level and contraction rate in β AR-stimulated cardiac myocytes. (A) Wild-type and PDE4BKO myocytes loaded with Fura-2 AM (5 μ M) were treated for the indicated times with Iso (10 nM). The average ratio R/R_0 ($R = \text{F340}/\text{F380}$) is reported and the number of cells analyzed (n) includes data from three experiments. Data were analyzed using a two-way ANOVA. Error bars correspond to the s.e.m. (B) The contraction rate of wild-type and PDE4BKO myocytes was measured before and 5 min after β AR stimulation with 10 nM Iso. The bar graphs represent the mean \pm s.e.m. of five experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (unpaired Student's t -test).

associates with β -arrestin (Lynch et al., 2005), and PDE4D9 co-immunoprecipitates with the β_2 AR (De Arcangelis et al., 2009). These distinct interactions and regulations of individual PDE4 variants by the two β -adrenergic receptors demonstrate that cAMP hydrolysis during catecholamine stimulation is not uniformly activated throughout the cell. Instead, dissipation of the cAMP signal in different subdomains occurs at different rates depending on the PDE4 subtype activated by different receptors.

β_1 AR-dependent cAMP signals in cardiac myocytes are far reaching and prolonged, whereas β_2 AR signals are transient and limited to restricted subdomains (Nikolaev et al., 2006). This difference might explain the finding that PDE4B is activated by β_1 AR but not β_2 AR activation. Localized β_2 AR signals might not reach and activate PDE4B, which in turn does not affect β_2 AR-dependent cAMP accumulation. We have previously reported that the chronotropic effects of β_2 AR stimulation are not affected in PDE4BKO neonatal cardiac myocytes, reinforcing the present observation of the lack of functional interaction between PDE4B and β_2 AR. One additional explanation of our finding is that the two signaling components are located in two separate membrane domains. β_2 AR has been localized to lipid rafts where adenylyl cyclase (AC)5, AC6 and AKAP150 (Gorelik et al., 2013; Xiang et al., 2002) are located, but also in T-tubules (Nikolaev et al., 2010). Our data suggest that PDE4B is located instead in the vicinity of the β_1 AR and in close proximity to a pool of PKA that regulates LTCC and RyR2, all components present in the dyad of neonatal and adult cardiac myocytes (Bers, 2008). It is possible that β_1 AR and β_2 AR are mutually exclusive in their localization in the dyad space and that PDE4B is present exclusively in the domain where β_1 AR is present. The presence of different pools of LTCC coupled to β_1 AR or β_2 AR have been proposed (Nichols et al., 2010) but the mechanisms underlying this sequestration are at present unknown.

Surprisingly, PDE4B ablation does not lead to spillover of cAMP to other compartments that are distant from the membrane, as one would expect if this PDE constitutes the primary barrier to cAMP diffusion. Absence of detectable changes in PKA phosphorylation in domains close to the myofilaments where MyBP-C and TnI are phosphorylated or close to the PLB-SERCA complex, are consistent with this view. After removal of PDE4B, this compartment also remains inaccessible to either β_2 AR or PGE2 signals, again suggesting the presence of a barrier not dependent on PDE4B. Such hindered diffusion might include physical barriers generated by the network of SR membranes or the actin cytoskeletal meshwork present below the membrane. More importantly, the presence of cAMP-binding proteins that slow the diffusion rate may be crucial to stabilizing this compartment. It is also possible that the pool of cAMP affected by PDE4B, although functionally important, is small compared to the total cAMP and that spill over to a much larger pool would be difficult to detect. A role of other PDEs such as PDE4D in limiting diffusion should also be considered.

In summary, our findings reveal a highly specialized function of PDE4B in β AR signaling of cardiac myocytes. The regulation of this enzyme is likely relevant to the pathophysiology of heart failure. We demonstrate here that ablation of PDE4B leads to aberrant phosphorylation of RyR2 and LTCC phosphorylation (but not of PLB), affecting Ca^{2+} handling, similar to that observed in heart failure (Chen et al., 2002; Lehnart et al., 2005; Schröder et al., 1998). Importantly, a decrease in PDE4B expression and activity is detected during cardiac hypertrophy (Abi-Gerges et al., 2009). Thus, PDE4B dysregulation might be a molecular adaptive change that contributes to arrhythmias in the failing heart.

MATERIALS AND METHODS

Reagents

β AR ligands Isoproterenol (Iso), ICI-118551 (ICI), CGP-20712A (CGP) and Propranolol, the pan-PDE inhibitor IBMX (3-Isobutyl-1-methylxanthine) and the PDE4-selective inhibitor Rolipram were from Sigma-Aldrich (St. Louis, MO, USA). The adenylyl cyclase activator Forskolin (Fsk) was from Tocris Bioscience (Bristol, UK). The PKA inhibitor H89 and the EP2/4 agonist prostaglandin E2 (PGE2) were from Cayman Chemicals (Ann Arbor, MI, USA). The selective PDE4B inhibitor [3-[[[5-(3-fluorophenyl)-2-pyrimidinyl]amino]4-ethoxy-phenyl]-4-morpholinyl-methanone (GSK16) was synthesized by Grünenthal GmbH (Aachen, Germany), according to a poster presentation by Mitchell C. et al. (GlaxoSmithKline) at the 46th International Conference on Medicinal Chemistry (RICT 2010, Reims, France). The Fura-2 AM Ca^{2+} indicator was from Invitrogen (Grand Island, NY, USA). Antibodies against the following proteins were used in this study: phospho-phospholamban (P-PLB, Ser16) and cardiac phospho-troponin I (P-TnI, Ser23/24), both from Cell Signaling Technology (Danvers, MA, USA), cardiac phospho-myosin binding protein C (P-MyBP-C, Ser282), from Alexis Biochemicals (Farmingdale, NY, USA) and calsequestrin (CSQ), from Affinity BioReagents (Golden, CO, USA). L-type Ca^{2+} channel pore forming subunit ($\text{Ca}_v1.2$) phosphorylation at Ser1928 was detected using the anti-CH3-P antibody (De Jongh et al., 1996; Hulme et al., 2006). The anti-phospho-ryanodine receptor (P-RyR2, Ser2808) antibody was a kind gift from Andrew Marks (Columbia University, New York, USA) (Wehrens et al., 2004). Pan-selective antibodies against PDE4A (AC55) and PDE4D (M3S1) were as described previously (Richter et al., 2005). Rabbit antibody 113-4, raised against the C-terminus of PDE4B, was used to detect PDE4B. Antibodies against PDE4B1 and PDE4B3 were from Fabgennix (Frisco, TX, USA). Horseradish-peroxidase-conjugated anti-rabbit IgG was from Amersham (Pittsburg, PA, USA).

Isolation of neonatal cardiac myocytes and adenoviral infection

Neonatal cardiac myocytes (NCMs) were isolated from the excised hearts of 1–2-day-old neonatal mice as described previously (Richter et al., 2008). They were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Nu Serum IV (BD Biosciences, San Jose, CA, USA), 5% fetal bovine serum, 1 mM glutamine, 30 $\mu\text{g}/\text{ml}$ penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 3 mM Hepes and 1 \times ITS media supplement (Gibco, Grand Island, NY, USA) on plates precoated with 10 $\mu\text{g}/\text{ml}$ laminin (Invitrogen). All cells were cultured at 37°C and under a 5% CO_2 atmosphere. All experiments were carried out on day 3 of culture. All animal studies were performed according to the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of the University of California San Francisco. For expression of the FRET sensors Epac2-CYTO or Epac2-PM, NCMs cultured on laminin-coated glass coverslips for 48 h were infected with the respective adenoviruses at a multiplicity of infection of 500.

Measurement of local cAMP levels using the Epac2-CYTO and Epac2-PM FRET sensors

For FRET (fluorescence resonance energy transfer) microscopy, glass coverslips were placed in a modified Sykes-Moore Chamber and kept in 500 μl of Locke's medium (5 mM HEPES pH 7.4, 154 mM NaCl, 5.6 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 3.6 mM NaHCO_3 , 5 mM glucose, 0.05% BSA) at 37°C. Images were acquired with a Nikon TE2000 inverted fluorescence microscope using a 60 \times fluorescence objective. CFP (donor) fluorescence was viewed by exciting at 430–455 nm and measuring emission at 470–490 nm. YFP (acceptor) fluorescence was viewed by exciting at 500–520 nm and measuring emission at 535–565 nm. FRET was viewed by exciting at 430–455 nm (donor excitation) and measuring fluorescence at 535–565 nm (acceptor emission). Images were acquired every 20 s. Background and bleedthrough were subtracted from FRET images to obtain corrected FRET images using MetaMorph software (Molecular Devices,

Sunnyvale, CA, USA) and average FRET intensity was measured directly in the corrected FRET images.

Preparation of protein extracts

Isolated NCMs were homogenized in an ice-cold buffer containing 150 mM NaCl, 20 mM HEPES (pH 7.4), 2 mM EDTA and 0.2 mM EGTA, supplemented with 10% glycerol, 0.2% Triton X-100, 1 μmol/l microcystin-LR (Cayman Chemicals) and Complete Protease Inhibitor Tablets (Roche Diagnostics, Pleasanton, CA, USA). Lysates were rotated at 4°C for 30 min followed by a 10-min centrifugation at 20,000 g and 4°C. Supernatants were either used directly for western blotting or subjected to immunoprecipitation (see below).

Immunoprecipitation of PDE4B from cell lysates

Immunoprecipitation of PDE4B was performed as described previously (Richter et al., 2011). In brief, cell and tissue extracts, prepared as described in 'Preparation of protein extracts', were precleared for 1 h using 50 μl protein-G-Sepharose (Invitrogen), followed by a second centrifugation at 20,000 g and 4°C for 10 min. Precleared detergent extracts were then immunoprecipitated using 10 μg of PDE4B antibody or 10 μg of control rabbit IgG for 2 h at 4°C.

PDE assay

The hydrolysis of cAMP by PDEs was measured according to the method of Thompson and Appleman as described previously (Richter and Conti, 2002). In brief, samples were assayed in a 200-μl reaction mixture containing 40 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, 1.4 mM β-mercaptoethanol, 1 μM cAMP, 0.75 mg/ml BSA and 0.1 μCi of [³H]cAMP for 20 min at 33°C. The reaction was terminated by heat inactivation in a boiling water bath for 1 min. The PDE reaction product 5'-AMP was then hydrolyzed by incubation of the assay mixture with 50 μg *Crotalus atrox* snake venom for 20 min at 33°C, and the resulting adenosine was separated by anion exchange chromatography using 1 ml AG1-X8 resin (Bio-Rad) and quantified by scintillation counting.

Measurement of intracellular Ca²⁺ level

NCMs cultured on laminin-coated glass coverslips for 48 h, were loaded with Fura-2 AM (5 μM, 30 min) then washed in Locke's medium. The coverslips were placed in a modified Sykes-Moore Chamber and kept in 500 μl of Locke's medium at 37°C. Images were acquired with a Nikon TE2000 inverted fluorescence microscope using a 20× objective. Fura-2 ratio (measured at 512 nm upon excitation at 340 nm and 380 nm) was recorded every 10 s using the MetaFluor software.

Measurement of myocyte contraction rate

Myocytes were cultured on laminin-coated cell culture dishes to obtain a uniformly beating syncytium. After 48 h in culture, the medium was replaced with serum-free medium, and experiments were carried out on day 3 of culture. To measure the myocyte contraction rate, the culture dish was placed on an inverted microscope for 5 min to equilibrate. Stacks of images were then stream acquired at 1-min intervals for 5 min before, and for another 10 min after addition of Iso (10 nM). The resulting images were analyzed with MetaMorph Object-Track to determine beat frequency.

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Competing interests

The authors declare no competing interests.

Author contributions

D.M. and W.R. performed the experiments and analyzed the data. D.M., W.R. and M.C. designed the study. D.M., W.R., R.E.W., W.A.C. and M.C. interpreted the data and prepared the manuscript.

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Supplementary material

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.140251/-DC1>

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