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Permalink
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Journal
Proceedings of the National Academy of Sciences of the United States of America, 112(7)

ISSN
0027-8424

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Publication Date
2015-02-17

DOI
10.1073/pnas.1419992112

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A CaMKII/PDE4D negative feedback regulates cAMP signaling

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Edited by Joseph A. Beavo, University of Washington School of Medicine, Seattle, WA, and approved January 13, 2015 (received for review October 18, 2014)

cAMP production and protein kinase A (PKA) are the most widely studied steps in β-adrenergic receptor (βAR) signaling in the heart; however, the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is also activated in response to βAR stimulation and is involved in the regulation of cardiac excitation-contraction coupling. Its activity and expression are increased during cardiac hypertrophy, in heart failure, and under conditions that promote arrhythmias both in animal models and in the human heart, underscoring the clinical relevance of CaMKII in cardiac pathophysiology. Both CaMKII and PKA phosphorylate a number of protein targets critical for Ca^{2+} handling and contraction with similar, but not always identical, functional consequences. How these two pathways communicate with each other remains incompletely understood, however. To maintain homeostasis, cyclic nucleotide levels are regulated by phosphodiesterases (PDEs), with PDE4s predominantly responsible for cAMP degradation in the rodent heart. Here we have reassessed the interaction between cAMP/PKA and Ca^{2+}/CaMKII signaling. We demonstrate that CaMKII activity constrains basal and βAR-activated cAMP levels. Moreover, we show that these effects are mediated, at least in part, by CaMKII regulation of PDE4D. This regulation establishes a negative feedback loop necessary to maintain cAMP/CaMKII homeostasis, revealing a previously unidentified function for PDE4D as a critical integrator of cAMP/PKA and Ca^{2+}/CaMKII signaling.

PDE | CaMKII | cAMP | β-adrenergic signaling | cyclic nucleotide phosphodiesterase

During cardiac excitation-contraction coupling (ECC), Ca^{2+} elevation throughout the cell promotes myofilament sliding, which generates contractile force. This process is highly regulated by positive and negative regulatory circuits, the most critical being the sympathetic nervous system that acts via activation of the β-adrenergic (βAR)/cAMP/PKA signaling pathway. During βAR stimulation, PKA phosphorylates and activates key proteins involved in ECC and Ca^{2+} handling. These proteins include L-type Ca^{2+} channels and ryanodine receptors (RyR), leading to enhanced Ca^{2+} influx and consequent sarcoplasmic reticulum (SR) Ca^{2+} release; phospholamban (PLB), increasing SR Ca^{2+} uptake by the Ca^{2+} ATPase (SERCA), thereby accelerating cardiac relaxation; and contractile proteins, increasing cell contraction. Collectively, these events produce the typical inotropic and lusitropic effects of βAR stimulation (1).

This βAR/cAMP/PKA pathway is only one of the components involved in regulating cardiac function, however. Data accumulated in the last decade have revealed that Ca^{2+}/calmodulin-dependent kinase II (CaMKII) is equally important to the regulation of cardiac function under physiological and pathological conditions (2–7). Most of the functions distal to cAMP and PKA are regulated by CaMKII as well (8). Proteins critical for ECC are substrates for both PKA and CaMKII, and phosphorylation at different sites often produces similar changes in protein function. Whereas CaMKII is directly regulated by Ca^{2+}, its activity is indirectly regulated by cAMP (9). In response to βAR stimulation, Epac (Exchange Protein directly Activated by cAMP) activates CaMKII (10–14). This regulation is critical in pathophysiological conditions in which CaMKII expression and activation may be elevated, such as hypertrophy, heart failure, and arrhythmias (2, 3). Despite the wealth of data available, the exact mechanisms integrating CaMKII activity with cAMP/PKA signaling remain unclear.

An established concept in cell signaling is that freely diffusible cAMP is not distributed uniformly throughout the cell, but is compartmentalized to generate specificity and to allow PKA regulation in distinct subdomains (15). Phosphodiesterases (PDEs), the enzymes that degrade cAMP, have emerged as ubiquitous and important modulators of cAMP/PKA signaling in specific cellular compartments, including cardiac myocytes (16, 17). They are part of macromolecular complexes that include PKAs and A-kinase anchoring proteins (AKAPs). In contrast to the large body of data linking PDEs to PKA regulation, whether PDEs regulate CaMKII is unclear. We and others have shown that genetic ablation of PDE4s, the isoform responsible for cAMP degradation in the heart, disrupts ECC via PKA-mediated alteration in Ca^{2+} handling (18–20); however, the possibility that some of the effects are mediated by CaMKII has not been investigated.

For proper homeostasis, cAMP signals are constrained by feedback mechanisms required to tightly regulate cyclic nucleotide levels under basal or stimulated conditions, with the feedback regulation of PDE4s a preeminent example. PDE4s are activated by PKA phosphorylation, providing a negative feedback mechanism by which cAMP regulates its own level (21, 22). Given the cAMP-dependent, Epac-mediated CaMKII activation, we surmise that feedback mechanisms linking cAMP and CaMKII must be operating in cardiac myocytes as well. In the present study, we reassessed the interaction between cAMP signaling and CaMKII. We demonstrate that CaMKII activity constrains basal and βAR-activated cAMP signaling. These effects are mediated, at least in part, by CaMKII regulation of PDE4D, revealing an additional function of this enzyme as a critical integrator of cAMP/PKA and Ca^{2+}/CaMKII signaling.

Significance

Cardiovascular disease is the leading cause of death worldwide. A better understanding of the pathophysiological mechanisms governing such disease states as heart failure is essential to the identification of novel therapeutic targets. We have discovered a regulatory loop that coordinates the function of proteins and secondary messengers involved in cardiac contractility and the development of heart failure. This regulatory loop may be a novel target for pharmacologic intervention to restore cardiac homeostasis.

Author contributions: D.M. and M.C. designed research; D.M., W.R., and M.C. performed research; D.M. and M.C. analyzed data; and D.M. and M.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1419992112/-/DCSupplemental.

Results

CaMKII Suppresses cAMP Accumulation. To test the hypothesis that an integrated Ca^{2+} and cAMP signaling in cardiac myocytes involves CaMKII-dependent regulation of cAMP, we first tested whether manipulation of the CaMKII activity affects cAMP accumulation in mouse neonatal cardiac myocytes (NCMs) (Fig. 1). We monitored cAMP changes in the cytosol of NCMs using the fluorescence resonance energy transfer (FRET)-based sensor Epac2-camps (23). We found that CaMKII inhibition by 1 μM KN93 (Fig. 1*A*) caused a highly significant rise in cytosolic cAMP, whereas the inactive derivative KN92 (1 μM) produced no effect (Fig. 1 *A* and *B*). An increase in cAMP identical to that induced by KN93 was observed with the structurally unrelated compounds KN62 (1 μM) and autocamtide-2–related inhibitory peptide (AIP; 2 μM), excluding the possibility of off-target effects of the drugs used (Fig. 1*B*). A tonic CaMKII-dependent inhibition of cAMP was even more apparent when cells were stimulated with a βAR agonist. Along with increasing basal cAMP, KN93 pretreatment caused a major potentiation of the effect of the βAR agonist isoproterenol (Iso; 10 nM) on cAMP (Fig. 1*C*).

To confirm the increase in cAMP levels after CaMKII inhibition through an independent approach, we investigated PKA activation by monitoring the effects of KN93 on the phosphorylation state of PKA substrates. Consistent with the cAMP measurements, KN93 induced increased phosphorylation of PLB at Ser16 both under basal conditions and after Iso stimulation (Fig. 1*D*). If blockage of CaMKII causes an increase in cAMP and PKA activation, as detected with the FRET probe and PLB phosphorylation, this inactivation should affect Ca^{2+} handling in myocytes as well. Myocytes were loaded with Fura2-AM, and the effect of acute combined Iso plus KN93 treatment was tested. In myocytes in which CaMKII was inhibited, Iso produced a transient but significant increase in peak intracellular Ca^{2+} concentration compared with controls (*SI Appendix*, Fig. S1).

In a converse experiment, activation of CaMKII by pretreatment of NCMs with the Ca^{2+} channel agonist BayK8644 (1 μM) caused a decrease in Iso-stimulated cytosolic cAMP (*SI Appendix*, Fig. S2*A*) and PKA-dependent phosphorylation of PLB at Ser16 (*SI Appendix*, Fig. S2*B*). These experiments suggest that CaMKII suppresses cAMP accumulation in cardiac myocytes. Similar data were obtained when using mouse embryonic fibroblasts, indicating that the negative effects of CaMKII on cAMP are not restricted to cardiac myocytes (*SI Appendix*, Fig. S3).

We next determined whether the increase in cAMP levels after CaMKII inhibition is related to an increase in the rate of cAMP synthesis and/or a decrease in the rate of cAMP hydrolysis by PDEs. To this end, Iso-dependent cAMP synthesis was blocked by the addition of excess propranolol, a βAR antagonist (1 μM) (Fig. 1*E*), and the rate of cAMP decay was measured. Assuming complete blockage of adenylyl cyclase activation by propranolol, the rate of cAMP decay reflects the endogenous PDE activity. Under these experimental conditions, the $t_{1/2}$ of cAMP decay was reduced by ∼50% in the presence of KN93 (Fig. 1*F*), indicating that CaMKII inhibition is associated with a decrease in PDE activity. KN93 and KN62 do not exert their actions through direct inhibition of PDE4, as demonstrated by the fact that these compounds did not affect cAMP hydrolysis in a cell-free assay of PDE activity (*SI Appendix*, Fig. S4).

PDE4D Is the Target of CaMKII Regulation of cAMP. Multiple PDEs are expressed in cardiac myocytes, with PDE3 and PDE4 isoforms/subtypes the most abundant forms. To test the involvement of these PDE families, we assessed the effect of CaMKII inhibition in the presence or absence of cilostamide (1 μM), a selective PDE3

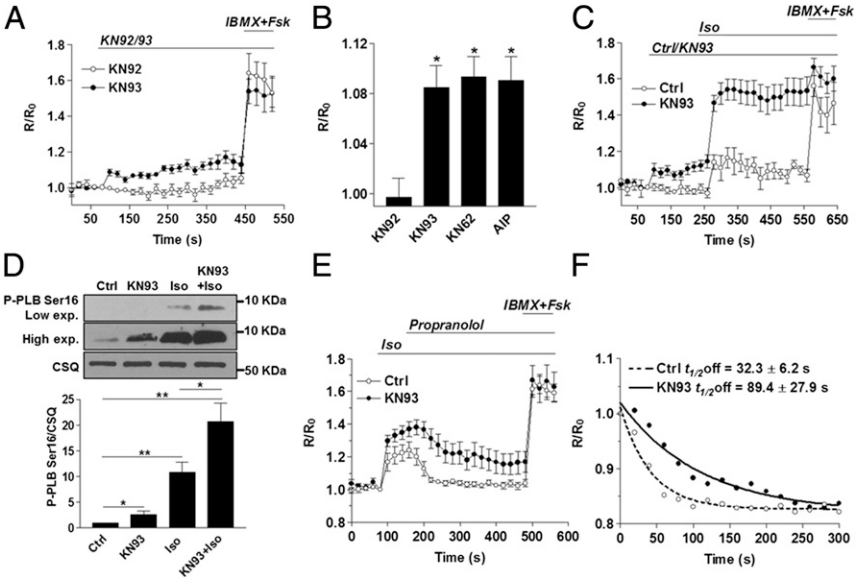

Fig. 1. CaMKII exerts a negative control on cAMP by regulating its hydrolysis. (*A*) Cardiac myocytes expressing Epac2-CYTO were treated for the indicated times with KN93 (1 μM; $n = 12$ cells) or the inactive compound KN92 (1 μM; $n = 6$). At the end of the recording, a broad-spectrum PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX; 100 μM) and forskolin (Fsk; 50 μM) were added to assess the maximum cAMP-producing capacity of the cell. The average R/R_0 ratio (R = CFP/FRET) is reported. $P < 0.01$, two-way ANOVA. (*B*) Maximal cAMP responses after treatment with KN92, KN93, KN62 (1 μM; $n = 10$), or AIP (2 μM; $n = 9$). Cells were analyzed as in *A*. $^*P < 0.05$; $^{**}P < 0.01$, Student t test. (*C*) Myocytes were treated for the indicated times with KN93 (1 μM; $n = 14$) or vehicle (Ctrl; $n = 10$) and Iso (10 nM), followed by IBMX + Fsk. $P < 0.001$, two-way ANOVA. (*D*) Detergent extracts prepared from myocytes treated for 5 min with vehicle (Ctrl), KN93 (1 μM) or Iso (10 nM), or pretreated for 5 min with KN93 followed by a 5-min treatment with Iso, were probed for PLB phosphorylation (P-PLB; Ser16). Calsequestrin (CSQ) served as a loading control. The ratios of the immunoblot intensity of the phosphorylated proteins over that of CSQ were normalized to control (Ctrl). $^*P < 0.05$; $^{**}P < 0.01$, Student t test. (*E*) Myocytes were pretreated for 5 min with KN93 (1 μM; $n = 13$) or vehicle (Ctrl; $n = 10$), then stimulated for the indicated times with Iso (10 nM). Propranolol (1 μM) was added to the cell at 80 s after the addition of Iso, followed by IBMX + Fsk. $P < 0.05$, two-way ANOVA. (*F*) Data from *E* were fitted with an exponential decay equation, and the average $t_{1/2}$off was calculated for the two conditions. $P < 0.05$, Student t test. In *A*–*F*, error bars correspond to the SEM, and data are from at least three experiments.

inhibitor, and rolipram (10 μM), a selective PDE4 inhibitor. Treatment with cilostamide did not affect the augmentation of cAMP levels by KN93; conversely, PDE4 inhibition with rolipram obliterates the effect of KN93 on cAMP accumulation (Fig. 2*A*). Consistent with these FRET data, rolipram also ablated the effect of KN93 on the phosphorylation state of PLB at Ser16 (Fig. 2*B*). More importantly, in PDE4D-deficient cells (PDE4DKO), KN93 had no effect on cAMP levels (Fig. 2*C* and *SI Appendix*, Fig. S3), or on the basal and Iso-stimulated phosphorylation of PLB at Ser16 (Fig. 2*D*). Myocytes deficient in PDE4B (PDE4BKO) behaved similarly to WT cells (*SI Appendix*, Fig. S5*A*). These experiments indicate that PDE4D is the PDE4 involved in the negative CaMKII regulation of cAMP signaling in myocytes.

CaMKII Phosphorylates and Activates PDE4D. It is well established that PDE4/PDE4D isoforms are regulated by posttranslational modifications, including phosphorylation by PKA and ERK2 (19, 21). To test whether CaMKII may directly phosphorylate and activate PDE4D, we assessed CaMKII or PKA phosphorylation on PDE4D immunoprecipitated from HEK293 cells in a cell-free system. CaMKII catalyzed phosphorylation of PDE4D to levels comparable to PKA (Fig. 3*A*). When different PKA phosphorylation-defective PDE4D mutants (21) were used in this assay, CaMKII still induced phosphorylation of the protein (Fig. 3*B*), opening the possibility that CaMKII phosphorylates PDE4D at sites distinct from PKA. A direct CaMKII phosphorylation of PDE4D was confirmed when using a purified recombinant PDE4D expressed in insect cells (*SI Appendix*, Fig. S7 *A* and *B*).

We previously reported that PKA phosphorylation of PDE4D is associated with a shift in electrophoretic mobility of the protein (21), owing to the negative charge introduced by the phosphorylation. We used this readout to determine whether CaMKII activation has any effect on the PDE4D endogenous to the myocytes. The Iso-induced shift in electrophoretic mobility of PDE4D was significantly accentuated after BayK8644 treatment (Fig. 4 *A* and *B*). This effect on mobility is related to CaMKII activation, because KN93 completely prevented the BayK8644-dependent shift (Fig. 4 *A* and *B*).

The phosphorylation of PDE4D by CaMKII should induce an increase in the hydrolytic activity of the enzyme. To determine whether CaMKII activation in an intact cell indeed causes an increase in PDE4D activity, we treated cardiac myocytes with the Ca^{2+} channel agonist BayK8644 (1 μM), subjected cell extracts to PDE4D-immunoprecipitation (IP), and then measured the activity recovered in the IP pellet. At the same time, we used the phosphorylation state of PLB at Ser17 to monitor the BayK8644-dependent activation of CaMKII (*SI Appendix*, Fig. S6). A small but highly significant increase in PDE4D activity after BayK8644 stimulation was observed (Fig. 4*C*). This effect was confirmed by cell-free incubation of recombinant PDE4D with CaMKII (*SI Appendix*, Fig. S7 *C* and *D*).

We next tested the effect of CaMKII on the βAR dose-dependent activation of PDE4D (Fig. 4*D*). When CaMKII is activated in cardiac myocytes, the dose-dependent activation of PDE4D by Iso is shifted to the left by ∼10-fold. Again this effect was entirely dependent on CaMKII activation in the intact cell, because KN93 effectively blocked the synergism between BayK8644 and Iso (*SI Appendix*, Fig. S8). Thus, under conditions in which Iso causes a submaximal effect, the activation of CaMKII markedly potentiates the βAR stimulation of PDE4D. This finding is consistent with the FRET data reported above, where KN93 had a larger effect on cAMP levels in the presence of Iso than on basal cAMP accumulation (Fig. 1*C*).

PDE4 Inhibition Activates CaMKII. In response to βAR stimulation, Epac activates CaMKII (9); activation of PDE4D by the kinase then functions as a negative feedback loop regulating CaMKII itself by controlling the access of cAMP to Epac. Indeed, the widely used Epac activator 8-pCPT-2-O-Me-cAMP-AM (007-AM) reproduces the increase in PDE4D activity, the shift of PDE4D, and increased phosphorylation of PLB at Thr17 seen with BayK8644 (*SI Appendix*, Fig. S9). An additional prediction of our model is that removal of the PDE4 component from the regulatory loop should cause activation of CaMKII. Indeed, this was the case when PLB phosphorylation at the CaMKII site (Thr17) or CaMKII autophosphorylation (Thr286) were used as readouts of CaMKII activation in myocytes (Fig. 5*A* and *SI Appendix*, Fig. S10). Several previous studies suggested that Epac controls CaMKII activity in a PKA-independent manner in cardiomyocytes (Fig. 6), and that this is accompanied by enhanced CaMKII target phosphorylation, including PLB at Thr17. Therefore, we tested whether the effects of PDE4 inhibition on

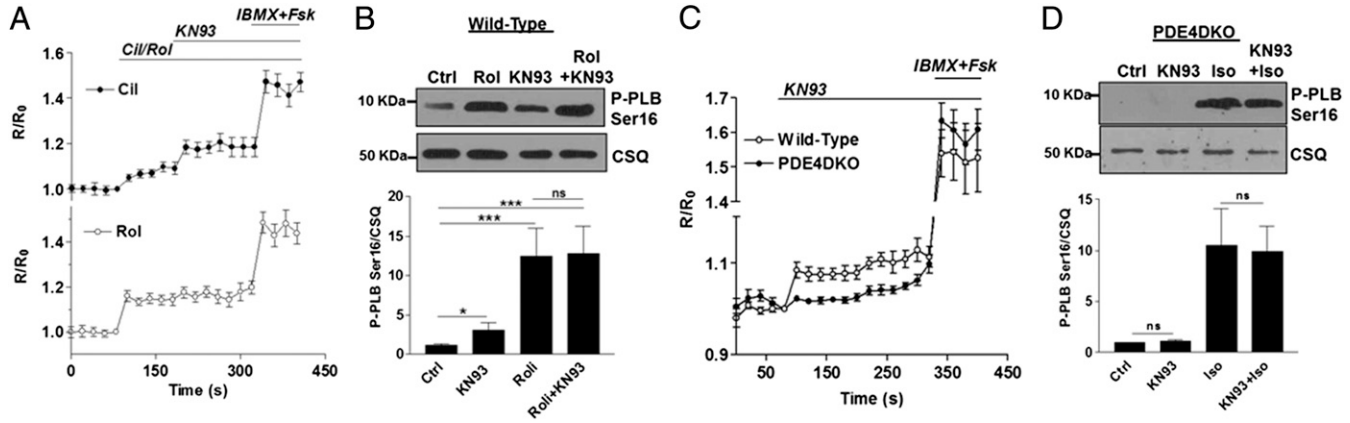

Fig. 2. PDE4D is involved in the CaMKII-dependent control of cAMP levels. (*A*) WT myocytes expressing Epac2-CYTO were treated for the indicated times with cilostamide (Cil; 1 μM; $n = 8$) or rolipram (Rol; 10 μM; $n = 18$), followed by KN93 (1 μM). IBMX (100 μM) and Fsk (50 μM) were added at the end of the recording. The average R/R_0 ratio (R = CFP/FRET) is reported. (*B*) Detergent extracts prepared from WT myocytes treated for 5 min with vehicle, Rol (10 μM), or KN93 (1 μM) or for 5 min with Rol followed by 5 min with KN93 were probed for PLB phosphorylation (P-PLB; Ser16). ns, not significant; $^*P < 0.05$; $^{***}P < 0.001$, Student t test. (*C*) WT ($n = 12$) and PDE4DKO ($n = 17$) myocytes expressing Epac2-CYTO were treated for the indicated times with KN93 (1 μM), followed by IBMX + Fsk. $P < 0.05$, two-way ANOVA. (*D*) Detergent extracts prepared from PDE4DKO myocytes treated for 5 min with vehicle (Ctrl), KN93 (1 μM), or Iso (10 nM), or for 5 min with KN93 followed by 5 min with Iso, were probed for PLB phosphorylation (P-PLB; Ser16). CSQ served as a loading control. The ratios of immunoblot intensity for the phosphorylated proteins over that for CSQ were normalized to control (Ctrl). ns, not significant; $^*P < 0.05$; $^{***}P < 0.001$, Student t test. In *A*–*D*, error bars are the SEM of data from three or more experiments.

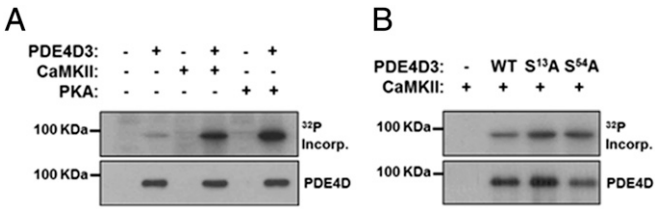

Fig. 3. CaMKII phosphorylates PDE4D in vitro. (*A*) PDE4D3 was overexpressed in HEK293 cells, immunoprecipitated with anti-PDE4D antibodies, and incubated with recombinant PKA or CaMKII in the presence of [γ-^{32}P]-ATP for 10 min. (*B*) WT and PKA phosphorylation-defective PDE4D3 mutants ($S^{13}A$, $S^{54}A$) were overexpressed in HEK293 cells, immunoprecipitated, and incubated with recombinant CaMKII in the presence of [γ-^{32}P]-ATP for 10 min. A representative experiment of the three or more experiments performed is reported.

CaMKII activity are indeed mediated by Epac, using the selective Epac2 inhibitor ESI05 (24). The rolipram-dependent increase in PLB phosphorylation at Thr17 was abolished by ESI05 (Fig. 5*B*). In line with the effect of rolipram, the Iso-dependent phosphorylation of PLB at Thr17 was clearly increased in PDE4DKO, but not PDE4BKO, myocytes compared with WT cells (Fig. 5*C* and *SI Appendix*, Fig. S5*B*), confirming that CaMKII becomes activated when PDE4D is inactivated.

Discussion

Although there is ample evidence that CaMKII is regulated by βAR activation, little information has been available on how CaMKII in turn modulates the βAR-dependent cAMP responses. The present study provides evidence for a novel regulation whereby the activities of CaMKII and PDE4D are integrated in a negative feedback loop. CaMKII activates PDE4D via phosphorylation, and PDE4D controls the access of cAMP to the signaling pathway leading to CaMKII activation. This feedback controls cAMP levels in cardiac myocytes, as documented by both cAMP and protein phosphorylation measurements. An important and widespread role in integrating cAMP and Ca^{2+} signaling is further supported by the effects of KN93 on Ca^{2+}, the CaMKII activation in PDE4DKO cardiac myocytes, and the finding that a similar feedback functions in fibroblasts.

Using cAMP single-cell FRET measurements, we show that compounds that inhibit CaMKII (25) induce a significant increase in cAMP in NCMs. Given their distinct structures and modes of action (26, 27), it is unlikely that the increase in cAMP levels is related to off-target effects of these drugs. We show that when used in a cell-free assay, none of these compounds inhibited the PDEs expressed in cardiac myocytes. In the converse experiment in which CaMKII was activated with the Ca^{2+} channel agonist, BayK8644 caused a significant decrease in cAMP. Taken together, these findings provide initial evidence that cAMP levels are regulated by CaMKII. The finding that phosphorylation of PLB at Ser16, as a proxy of PKA activity, followed an identical pattern provides independent confirmation that activation of CaMKII causes a decrease, or constrains the increase, in cAMP levels in cardiac myocytes.

The following findings demonstrate that the effects of CaMKII on cAMP are mediated by regulation of PDE4D. Measurement of the rate of cAMP decay in myocytes in the presence of the CaMKII inhibitor revealed a marked reduction in cAMP decay, indicating that the rate of cAMP hydrolysis in the intact cell is decreased when CaMKII is inactivated. The effect of CaMKII inhibition on cAMP was absent when PDE4 was inhibited with rolipram or when myocytes derived from PDE4D-null mice were used. These complementary findings indicate that PDE4D is the PDE functioning in concert with CaMKII. Whether Ca^{2+} and CaMKII manipulation also affect cAMP synthesis remains to be determined. Our data show a small effect of BayK8644 on the rate of cAMP synthesis. Adenylyl cyclases (AC) 5 and 6, which are expressed in cardiac myocytes, are inhibited by Ca^{2+} (28). In principle, it is possible that the Ca^{2+} channel agonist causes an increase in Ca^{2+}, which in turn inhibits the activity of the ACs; however, the finding that genetic or pharmacologic inhibition of PDE4D obliterates the effect of CaMKII strongly suggests that the major effect of CaMKII manipulation is at the level of cAMP hydrolysis. The possibility that BayK8644 activates cAMP hydrolysis by activating the Ca^{2+}-sensitive PDEs is unlikely, given the insensitivity to PDE1 to rolipram (29). The Ca^{2+} surge induced by BayK8644 may activate the Ca^{2+}-activated protein phosphatase (PP) type 2B (calcineurin)

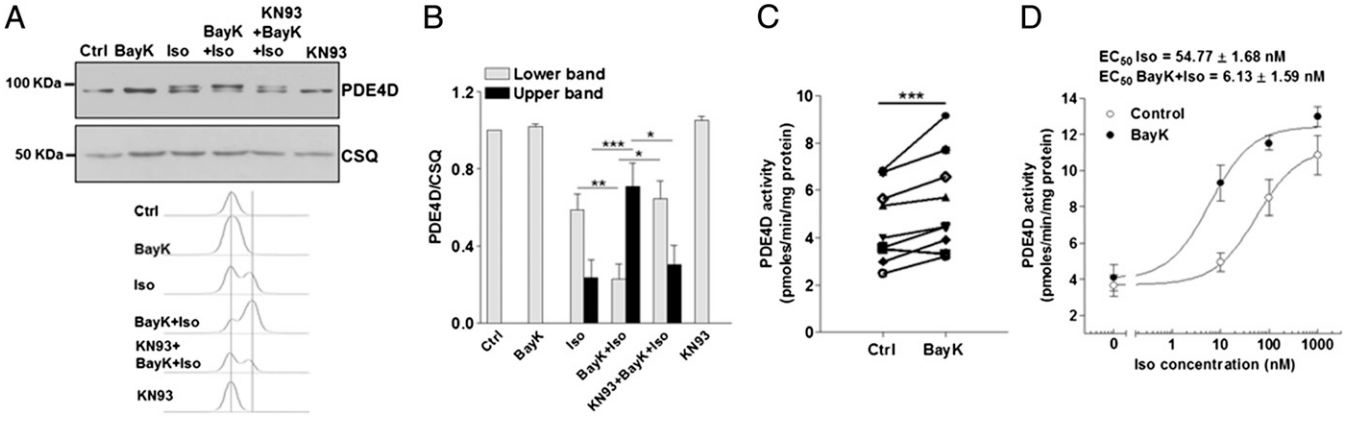

Fig. 4. CaMKII phosphorylates and activates PDE4D in vivo. (*A*) Detergent extracts prepared from WT myocytes treated for 5 min with vehicle (Ctrl), BayK (1 μM), Iso (1 μM), or KN93 (1 μM), or pretreated for 5 min with KN93 followed by 5 min with BayK and then a 5-min treatment with Iso, were probed for PDE4D. CSQ served as a loading control. A representative Western blot of five performed is included. The film was digitized by scanning, and the position of the immunoreactive bands was quantitated using ImageJ. The plot of the signal intensity for each lane of the blot is reported in arbitrary units. (*B*) The ratio of the immunoblot intensity for each PDE4D electrophoretic species over that for CSQ, normalized to the control condition (Ctrl). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, Student *t* test. (*C*) Detergent extracts prepared from myocytes treated for 5 min with or without BayK8644 (BayK; 1 μM) were subjected to IP with anti-PDE4D antibodies, and the PDE activity recovered in the IP pellets was measured. (*D*) Detergent extracts were prepared from myocytes treated for 5 min with or without BayK (1 μM), followed by a 5-min treatment with increasing concentrations of Iso. The extracts were then subjected to PDE4D IP. The activity recovered in the IP pellets is reported. The EC_{50} for each experiment was derived from fitting the data with a four-parameter logistic equation. $P < 0.001$, Student *t* test. *C* and *D* include data from three or more experiments. For *B* and *D*, error bars correspond to the SEM.

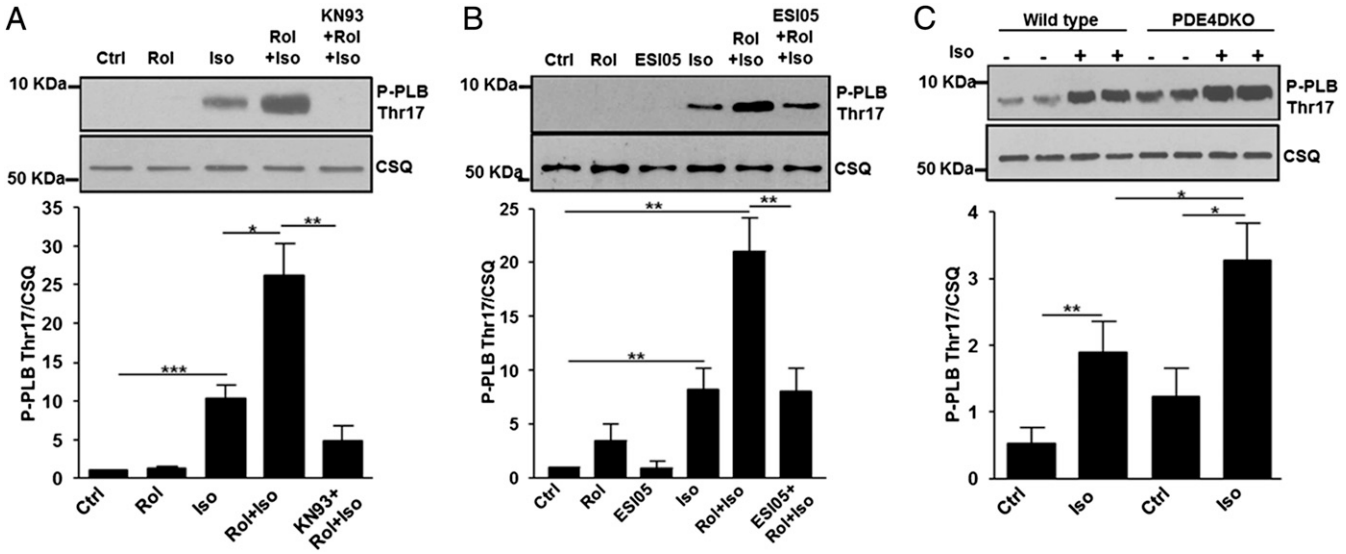

Fig. 5. PDE4 inhibition activates CaMKII. (*A* and *B*) Cardiac myocytes were treated for 5 min with vehicle (Ctrl), the Epac2 inhibitor ESI05 (10 μM), Rol (10 μM), Iso (10 nM), and/or KN93 (1 μM) (*A*) or ESI05 (*B*). (*C*) WT and PDE4DKO cells were treated for 5 min with Iso (10 nM). In *A*–*C*, total extracts were probed for PLB phosphorylation (P-PLB; Thr17). CSQ served as a loading control. A representative Western blot of the three or more performed is shown. The ratios of the immunoblot intensity of the phosphorylated proteins over that for CSQ were normalized to control cells (Ctrl). Error bars correspond to the SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, Student *t* test.

dephosphorylating inhibitor-1 and thereby activating PP1 (30, 31). PP1, in complex with key proteins of the ECC, such as L-type Ca^{2+} channels, RyR, or PLB, induces dephosphorylation of these substrates, thereby regulating cardiac electrophysiology and contraction (31). It is possible that the decrease in Ser16 PKA phosphorylation of PLB after BayK8644 treatment is related in part to Ca^{2+}-dependent dephosphorylation.

Three independent sets of data support the conclusion that PDE4D is a direct target of CaMKII phosphorylation. First, CaMKII phosphorylates PDE4D in a cell-free system to the same extent as PKA and increases its hydrolytic activity. Second, activation of CaMKII in the intact cell synergizes with PKA activation of PDE4D. Third, CaMKII and PKA have a synergistic effect on the mobility shift of PDE4D detected by SDS/PAGE. These findings support the hypothesis that CaMKII phosphorylation of PDE4D functions as permissive or hierarchical for PKA-mediated phosphorylation and PDE4D activation. Consistent with this view, the finding that CaMKII phosphorylates PDE4D when the PKA sites are mutated strongly suggests that the CaMKII phosphorylation sites are distinct from the PKA sites. Similarly, the synergistic effects on the mobility shift point to phosphorylation at two separate but interacting sites. It is possible that CaMKII phosphorylation causes a change in conformation of the PDE4D molecule that facilitates activation of the enzyme by PKA. When these findings are confirmed by mapping of the CaMKII phosphorylation sites, they will provide evidence of a novel regulatory domain in PDE4. Given the hypothesis that the PDE4 catalytic pocket is regulated by a "capping helix" of the upstream conserved region 2 (UCR2) (32), it will be important to determine the effect of this additional phosphorylation on the positioning of this critical domain.

We show that CaMKII activation with BayK8644 increases signaling downstream of CaMKII but represses cAMP/PKA-mediated responses, and, conversely, that CaMKII inhibition increases cAMP and PKA. Thus, under certain conditions, the CaMKII-dependent activation of PDE4D may serve to redistribute the βAR signals between the two kinases. The CaMKII/PDE4D feedback may play a role in those cases in which a switch from PKA to CaMKII signaling has been demonstrated. For instance, long-term βAR stimulation of rat cardiac myocytes in vitro increases contractility, Ca^{2+} loading, and Ca^{2+} sparks in the SR via CaMKII-dependent, but PKA-independent, mechanisms (33). In this case, the constitutively activated CaMKII may repress the PKA-mediated responses via activation of PDE4D. Moreover, the increase in cAMP that follows CaMKII inhibition is bound to impact ECC, as indicated by the increase in Ca^{2+} that we detected after KN93/Iso treatment, possibly confounding the interpretation of those experiments that use CaMKII inhibitors to dissect the function of this kinase on contractility during βAR activation (34).

An increase in basal and Iso-dependent PLB phosphorylation at Thr17 was present in PDE4DKO, but not PDE4BKO, myocytes. The effects of CaMKII inhibition on cAMP were negated in PDE4DKO, but not PDE4BKO or PDE3, inhibition. Thus, the pool of cAMP controlled by PDE4B does not have access to the PKA or Epac that directly or indirectly controls CaMKII. These findings strongly suggest that the CaMKII/PDE4D feedback loop is functioning in sequestered compartments of cardiac myocytes. Future experiments are needed to define the properties of this compartment and whether CaMKII, Epac, and

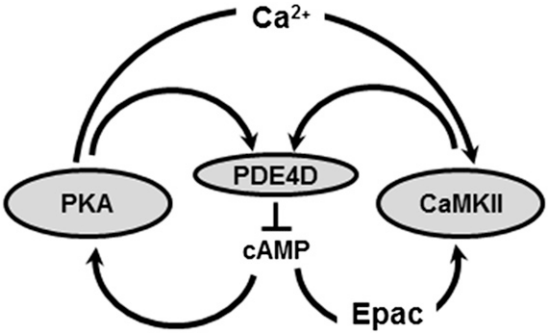

Fig. 6. Scheme of the proposed negative feedback regulation integrating cAMP, PDE4, and CaMKII in cardiac myocytes. cAMP in myocytes is controlled by a double-negative feedback mechanism in which an increase in cAMP activates PDE4D through PKA or Epac/CaMKII, which in turn promotes cAMP degradation.

BIOCHEMISTRY

PDE4D exist in a macromolecular complex organized by scaffold proteins. PDE4D is in complex with β-arrestin (35), and β-arrestins interact with CaMKII and Epac1 (36). Epac1 binds to PDE4D in vascular endothelial cells (37) and associates with the mAKAP/PKA complex in cardiac myocytes (38). Thus, the CaMKII/PDE4D regulatory loop may depend on the assembly of one of these macromolecular complexes. Even though Epac2 inhibition suppresses the effect of PDE4 inhibition, we cannot formally exclude the possibility that some of the effects are due to Epac1. It was recently reported that Epac1 regulates PLB phosphorylation and Ca^{2+} handling in the heart through a PLB/PKC pathway (39).

PDE4D gene inactivation causes two major cardiac phenotypes: increased propensity to arrhythmias and late-onset dilated cardiomyopathy (18, 20). In previous studies on PDE4DKO cells, we related an increased Ca^{2+} leakage to PKA-mediated phosphorylation of RyR (20); however, our data showing that PDE4D function is integrated with CaMKII strongly suggest that PDE4D inactivation also may disrupt cardiac homeostasis through dysregulation of CaMKII, and that this chronic activation in turn affects RyR-mediated Ca^{2+} leakage. The loss of PDE4D should cause aberrant cAMP that activates PKA and Epac2, both of which cause CaMKII activation. Indeed, we found increased PLB phosphorylation at the CaMKII site in PDE4DKO myocytes in basal conditions and after βAR stimulation. Genetic manipulation of CaMKII and PDE4D should clarify the long-term effect of this feedback.

In summary, our data strongly support the conclusion that feedback involving CaMKII and PDE4D is operating in a cell. This feedback regulation likely plays a critical role in cell homeostasis, including in cardiac myocytes, providing a new paradigm for understanding how Ca^{2+} and cAMP signaling are integrated. Given the fact that CaMKII is a point of convergence of multiple pathways, activation of PDE4D distal to this kinase may be useful for explaining other signaling feedbacks as well; for instance, PDE4 activity is required for maximal *N*-formyl-methionine-leucine-phenylalanine (fMLP)-dependent reactive oxygen species (ROS) production (40, 41), and ROS activate CaMKII (42) and PDE4D (43). Thus, the CaMKII/PDE4 feedback may be involved in regulating additional pathways implicated in pathophysiological conditions in the heart and ROS-mediated cell injury during inflammation.

Materials and Methods

Detailed information on reagents, cAMP and Ca^{2+} measurements, cell cultures, and biochemical assays is provided in *SI Appendix, Materials and Methods*.

ACKNOWLEDGMENTS. We are indebted to Dr. J. Pandit (Pfizer Central Research) for the generous gift of purified PDE4D. This study was funded by grants from the National Institutes of Health (HL0927088, to M.C.) and Fondation Leducq (06CVD02 cycAMP, to M.C.). D.M. is a recipient of a postdoctoral fellowship from the American Heart Association.

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