Phosphodiesterase Type 3A Regulates Basal Myocardial Contractility Through Interacting With Sarcoplasmic Reticulum Calcium ATPase Type 2a Signaling Complexes in Mouse Heart

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Rationale: **cAMP is an important regulator of myocardial function, and regulation of cAMP hydrolysis by cyclic nucleotide phosphodiesterases (PDEs) is a critical determinant of the amplitude, duration, and compartmentation of cAMP-mediated signaling. The role of different PDE isozymes, particularly PDE3A vs PDE3B, in the regulation of heart function remains unclear.**

Objective: **To determine the relative contribution of PDE3A vs PDE3B isozymes in the regulation of heart function and to dissect the molecular basis for this regulation.**

Methods and Results: **Compared with wild-type littermates, cardiac contractility and relaxation were enhanced in isolated hearts from PDE3A$^{-/-}$, but not PDE3B$^{-/-}$, mice. Furthermore, PDE3 inhibition had no effect on PDE3A$^{-/-}$ hearts but increased contractility in wild-type (as expected) and PDE3B$^{-/-}$ hearts to levels indistinguishable from PDE3A$^{-/-}$. The enhanced contractility in PDE3A$^{-/-}$ hearts was associated with cAMP-dependent elevations in Ca^{2+} transient amplitudes and increased sarcoplasmic reticulum (SR) Ca^{2+} content, without changes in L-type Ca^{2+} currents of cardiomyocytes, as well as with increased SR Ca^{2+}-ATPase type 2a activity, SR Ca^{2+} uptake rates, and phospholamban phosphorylation in SR fractions. Consistent with these observations, PDE3 activity was reduced ≈8-fold in SR fractions from PDE3A$^{-/-}$ hearts. Coimmunoprecipitation experiments further revealed that PDE3A associates with both SR calcium ATPase type 2a and phospholamban in a complex that also contains A-kinase anchoring protein-18, protein kinase type A-RII, and protein phosphatase type 2A.**

Conclusions: **Our data support the conclusion that PDE3A is the primary PDE3 isozyme modulating basal contractility and SR Ca^{2+} content by regulating cAMP in microdomains containing macromolecular complexes of SR calcium ATPase type 2a–phospholamban–PDE3A. (*Circ Res*. 2013;112:289-297.)**

Key Words: cAMP ■ calcium regulation ■ contractility ■ phosphodiesterase type 3A knock-out mice ■ sarcoplasmic reticulum calcium ATPase type 2a

Heart function is tightly regulated by the sympathetic-driven β-adrenergic system via alterations in the activity of cAMP-dependent protein kinase A (PKA).[1] The effects of PKA are finely tuned through A-kinase anchoring proteins (AKAPs) that recruit PKA into multiprotein complexes containing many signaling molecules, including 3′,5′-cyclic nucleotide phosphodiesterases (PDEs) and protein phosphatases, as well as end-effector molecules.[1,2] PDEs are a unique class of enzymes responsible for the degradation of cAMP, the primary molecule regulating PKA. PDEs are divided into 11 gene families, with many of the 21 individual PDE genes producing multiple protein products via alternative mRNA splicing or utilization of different transcription initiation sites. Recent studies have established that PDEs assemble in an isoform-specific manner into specialized macromolecular complexes within discrete functional compartments, thereby

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Non-standard Abbreviations and Acronyms	
AKAP	A-kinase anchoring protein
ICaL	L-type calcium current
LVDP	left ventricular developed pressure
PDE3A	phosphodiesterase type 3A
PLN	phospholamban
PKA	protein kinase type A
RyR2	cardiac ryanodine receptor
SR	sarcoplasmic reticulum
SERCA2a	sarcoplasmic reticulum calcium ATPase type 2a

allowing for precise spatiotemporal control of cAMP/PKA signaling.[3,4]

Murine hearts express genes from several PDE families. Although PDE8A recently was shown to regulate the acute actions of β-receptor stimulation,[5] PDE3 and PDE4 isoforms have been considered traditionally as the primary PDEs involved in the regulation of cardiac contractility in mice and other species.[6] In murine myocardium, both PDE3 and PDE4 activity regulate baseline Ca^{2+} transients and myocardial contractility by modulating cAMP/PKA–dependent sarcoplasmic reticulum (SR) Ca^{2+}-ATPase type 2a (SERCA2a pump) activity in microdomains that do not appear to contain ryanodine receptors or L-type Ca^{2+} channels.[7–9] Studies in humans also have established that PDE3 inhibitors markedly enhance myocardial contractility, relaxation, and diastolic function,[6,10] which have been linked to β-receptor–dependent and cAMP/PKA–dependent increases in SR Ca^{2+} uptake[11] via PKA-dependent phosphorylation of phospholamban (PLN),[12] although β-receptor–dependent effects on L-type Ca^{2+} channels have been reported also in rats.[13] These observations are consistent with a model wherein cAMP compartmentalization and Ca^{2+} transients are regulated by PDE3 enzymes within microdomains containing SERCA2a, as well as possibly other regulatory proteins.[2–4,14] Although PDE3 inhibitors provide short-term benefit in heart transplant patients and end-stage heart failure patients, particularly when responses to β-adrenergic receptor agonists are lacking, chronic administration of PDE3 inhibitors increases mortality.[15,16] Despite these cardiovascular side effects, PDE3 inhibitors (such as cilostazol), nevertheless, are used for treating intermittent claudication, a peripheral vascular disease.[17]

The molecular basis for the myocardial effects of PDE3 inhibitors remains unclear, with different studies suggesting that the major cardiac PDE3 isozyme is either PDE3A[18] or PDE3B.[19] Because currently available PDE3 inhibitors do not discriminate between PDE3A and PDE3B, it has been challenging to dissect the relative roles for PDE3A or PDE3B in the myocardium. In this study, we used PDE3A$^{-/-}$ mice[20] and PDE3B$^{-/-}$ mice[21] to better-characterize the isoform dependence for the regulation of myocardial contractility by PDE inhibitors. Our studies establish that PDE3A, not PDE3B, regulates baseline contractility in murine myocardium by cAMP-dependent modulation of Ca^{2+} transients, SERCA activity, and PLN phosphorylation in SERCA2a-containing SR microdomains of ventricular cardiomyocytes.

Methods

Animals

Age-matched (8–16 weeks old) wild-type (WT) and PDE3A$^{-/-}$ littermates on a C57BL6/J background[20] were used for experiments in this report. For some experiments, age-matched WT C57BL6 mice were purchased from Jackson Laboratories. Protocols for mouse generation and maintenance were approved by the National Heart, Lung, and Blood Institute Animal Care and Use Committee and the Canadian Council of Animal Care. The animal experimental protocols are in accordance with *Guide for the Care and Use of Laboratory Animals*

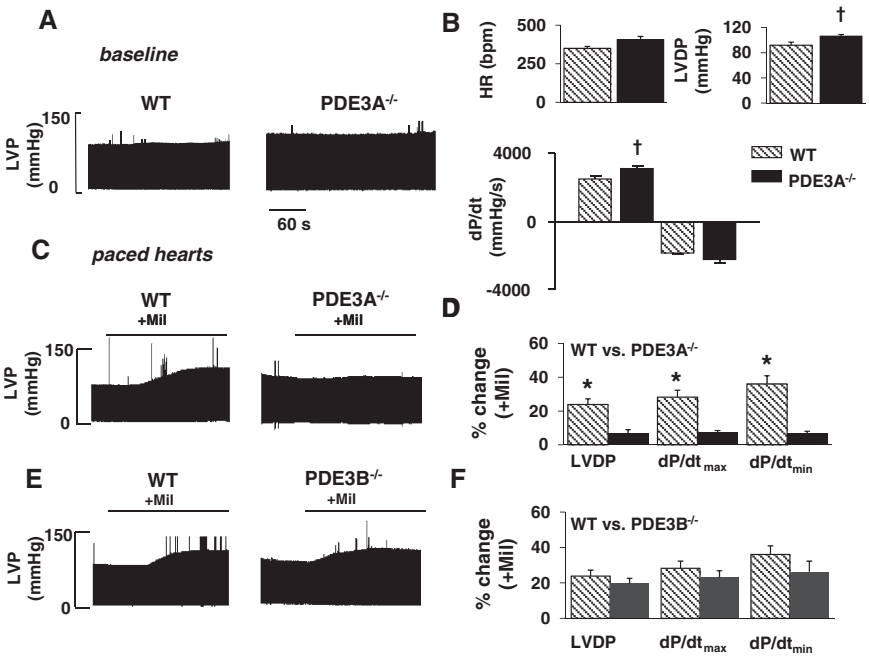

Figure 1. Phosphodiesterase (PDE) 3A–deficient hearts exhibit increased left ventricular developed pressure (LVDP) and dP/dt$_{max}$, but are not responsive to PDE3 inhibition with milrinone (Mil). A, Baseline left ventricular pressure measurements (after 20 minutes of equilibration period) in wild-type (WT) and PDE3A$^{-/-}$ hearts. **B**, Mean data comparing baseline cardiac function between WT (n=5) and PDE3A$^{-/-}$ (n=5) hearts. **C**, Pressure recordings in paced WT and PDE3A-deficient hearts during Mil infusion. **D**, Cardiac function changes in paced WT (n=5) and PDE3A$^{-/-}$ (n=5) hearts induced by Mil. **E**, Pressure measurements in paced WT and PDE3B-deficient hearts during Mil infusion. **F**, Cardiac function changes in paced WT (n=3) and PDE3B$^{-/-}$ (n=3) hearts after Mil treatment. Hearts were paced at rates (8 Hz) just above the intrinsic heart rates (HRs) observed after Mil treatment. dP/dt$_{max}$ and dP/dt$_{min}$ indicates maximum and minimum rates of change of pressure development, respectively. *P<0.05 vs baseline conditions; †P<0.05 vs WT control.

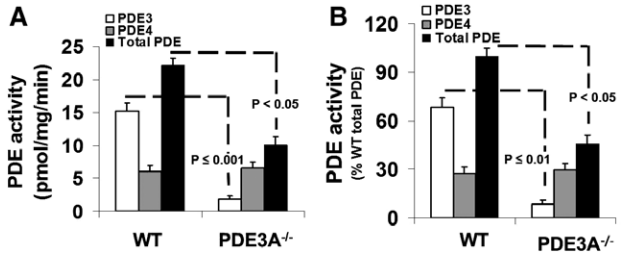

Figure 2. Total phosphodiesterase (PDE) and PDE3 activities are decreased in sarcoplasmic reticulum (SR) microsomes isolated from PDE3A$^{-/-}$ hearts. PDE activity in wild-type (WT) and PDE3A$^{-/-}$ cardiac SR fractions, expressed as (**A**) specific activity (pmol cAMP hydrolyzed/min per mg) or (**B**) a percentage of the total PDE activity in WT hearts. Total PDE activity was determined in 3 independent SR microsomal preparations using cilostamide to inhibit PDE3, rolipram to inhibit PDE4, and IBMX to inhibit total PDE activity. **P*<0.001 vs WT; ***P*<0.05 vs WT total PDE activity.

(National Institutes of Health Publication, revised 1996, No. 86-23). Detailed Methods for the experiments presented and discussed in this report are included in the Online Data Supplement.

Results

We started by measuring cardiac contractility in isolated ex vivo Langendorff hearts because PDE3 inhibition affects vascular resistance, which complicates the assessment of intrinsic cardiac function in intact mice.[22,23] As summarized in Figure 1A and 1B and in Online Table I, isolated hearts from PDE3A$^{-/-}$ mice showed elevated (*P*<0.05; n=5) contractility as assessed by left ventricular developed pressure (LVDP=106.5±2.6 mm Hg) and the maximum (positive) time derivative of LVDP (+dP/dt$_{max}$=3080±193 mm Hg/s) compared with WT (2499±101 mm Hg/s). We next explored the effects of PDE3A inhibition on contractility. Because PDE3 inhibition elevates heart rate in WT mice,[6] which influences contractility, we performed these studies on externally paced isolated hearts (via right atria) at rates (8 Hz) that were slightly above the heart rates observed (6.6±0.4 Hz) after PDE3 inhibition. Treatment with 10 µmol/L milrinone (Mil), which selectively inhibits both PDE3A and PDE3B,[22] had no effect on LVDP (*P*=0.71; n=5) or +dP/dt-$_{max}$ (*P*=0.55; n=5) in paced PDE3A$^{-/-}$ hearts but increased (*P*<0.001; n=5) these parameters in WT hearts (Figure 1C and 1D; Online Table I). Mil also had no effect on LVDP and +dP/dt$_{max}$ in unpaced PDE3A$^{-/-}$ hearts. In contrast, LVDP and +dP/dt$_{max}$ in PDE3B$^{-/-}$ hearts were indistinguishable from WT (*P*>0.477) before or after Mil treatment (Figure 1E and 1F). These functional studies support the conclusion that PDE3A is the primary PDE3 isozyme regulating baseline cardiac contractility. Consistent with this conclusion, Figure 2 shows that total PDE3 activity is ≈8-fold lower in SR fractions from mice lacking PDE3A (ie, 1.8±1.3 pmol/mg per minute in PDE3A$^{-/-}$ SR fractions compared with 15.1±1.8 pmol/mg per minute in WT), which is similar to results in heart homogenates.[20] Furthermore, the absolute differences in specific PDE3 activity were indistinguishable (*P*=0.493) from the absolute differences in total PDE activity between these groups (10.1±1.2 pmol/mg per minute in PDE3A$^{-/-}$ vs 22.5±1.1 pmol/mg per minute in WT). Although it is possible that the loss of PDE3A leads to compensatory changes in other PDE isoforms,[24] no changes in PDE4 activity (another major cardiac PDE in mice) were observed between PDE3A$^{-/-}$ and WT SR fractions (Figure 2).

To determine the cellular mechanisms mediating the effects of PDE3A on cardiac contractility, we simultaneously recoded Ca^{2+} transients and L-type Ca^{2+}channel currents ($I_{Ca,L}$) in isolated cardiomyocytes. Consistent with our isolated heart studies, Figure 3 and Online Table II establish that Ca^{2+} transients ($\Delta F/F_O$=3.6±0.6) were elevated in PDE3A$^{-/-}$ cardiomyocytes (*P*<0.05), along with trends toward faster (*P*=0.08) decay (τ=142±4.4 ms; n=9) compared with WT

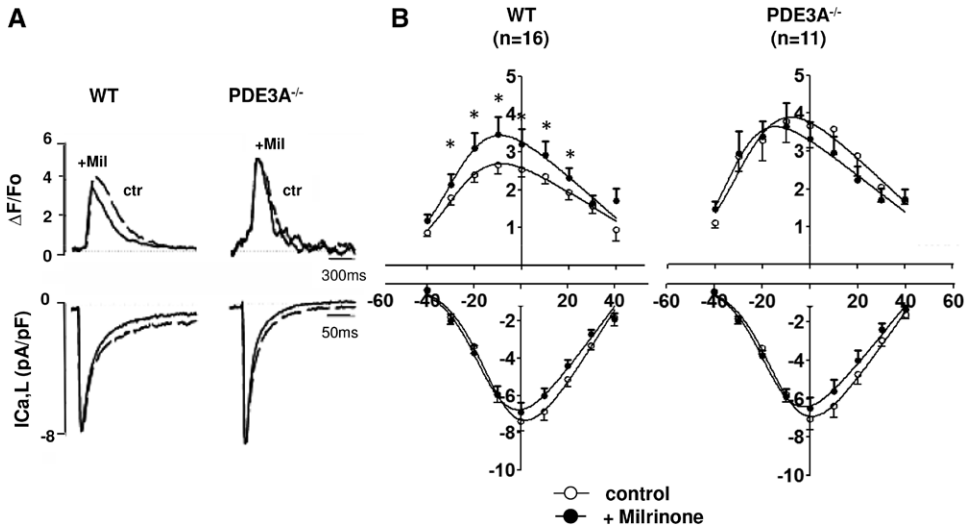

Figure 3. Effects of phosphodiesterase (PDE) 3A ablation on Ca2+ transients and L-type calcium current (ICaL). A, Raw traces of simultaneous Ca^{2+} transients (**top**) and $I_{Ca,L}$ (**bottom**) measured at 0 mV. The holding potential was −85 mV, and a 200-ms depolarization ramp to −45 mV was added before the depolarization step to 0 mV. The measurements were made 8 minutes after membrane rupture to permit full equilibration of the Fluo-3 dye. Either milrinone (Mil) or vehicle (control) was added for an additional 8 minutes before measurements of I_{CaL} and Ca^{2+} transients were made. **B**, Mean data illustrating effects of PDE3A ablation on Ca^{2+} transient amplitudes (**top**) and I_{CaL} densities (**bottom**) in wild-type (WT; n=16) and PDE3A$^{-/-}$ (n=9) cardiomyocytes with protocols as in (**A**), except that depolarizations were varied from −40 to +30 mV (see Online Data Supplement Methods). **P*<0.05 vs control in same group; †*P*<0.05 between groups.

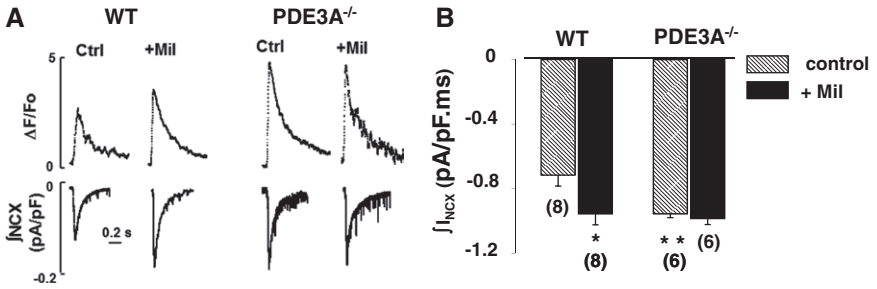

Figure 4. Phosphodiesterase (PDE) 3A ablation increases sarcoplasmic reticulum (SR) Ca^{2+} content and SR Ca^{2+} ATPase type 2a (SERCA2a) function. A, Representative traces depicting Ca^{2+} release from the SR (transients at the **top**) and accompanying I_{NCX} (**bottom**) induced by application of 20 mmol/L caffeine to wild-type (WT) and PDE3A$^{-/-}$ cardiomyocytes, and then after perfusion with 10 μmol/L milrinone (Mil). **B**, Average data illustrating the effects of PDE3 inhibition with 10 μmol/L Mil (+Mil) or PDE3A ablation on SR Ca^{2+} content as gauged by integrating I_{NCX} (n values indicated). *P<0.05 vs WT control.

($\Delta F/F_O$=2.5±0.2 and τ=158±9.7 ms; n=16). In contrast, $I_{Ca,L}$ properties (maximal conductance [G_{max}], the half-maximal activation voltage [$V_{1/2}$], or inactivation kinetics of $I_{Ca,L}$) did not differ between PDE3A$^{-/-}$ and WT cardiomyocytes (Online Table II) even after maximal activation of cAMP/PKA[25] using a combination of 100 nmol/L isoproterenol plus 100 μmol/L of 3-isobutyl-1-methylxanthine (Online Figure I). Increased Ca^{2+} transients without $I_{Ca,L}$ alterations in the PDE3A$^{-/-}$ cardiomyocytes were observed at all voltages (Figure 3), demonstrating that PDE3A ablation enhances excitation–contraction coupling efficiency[26] at all voltages (Online Figure II). Importantly, Mil increased (P<0.001; n=16) Ca^{2+} transients and accelerated (P=0.026; n=16) their decay in WT, but not in PDE3A$^{-/-}$, cardiomyocytes (P=0.219; n=9) without effecting $I_{Ca,L}$ in either group (Figure 3B and Online Table II). A definitive role for cAMP in the differences between WT and PDE3A$^{-/-}$ cardiomyocytes was established by dialyzing myocytes with the selective cAMP antogonist, Rp-cAMPS, which had no effect (P=0.117, n=7) on Ca^{2+} transients in WT cardiomyocytes, but reduced the amplitudes (P<0.001; n=7) and prolonged the relaxation times (P<0.05; n=7) of Ca^{2+} transients in PDE3A$^{-/-}$ cardiomyocytes to the levels indistinguishable from the amplitudes (P=0.151) and relaxation times (P=0.635) in WT cardiomyocytes with or without Rp-cAMPS dialysis (Online Figure III and Online Table II). To establish whether the cAMP-dependent elevations in Ca^{2+} transients originated from altered SR Ca^{2+} levels, we measured time-integrated Na^+/Ca^{2+} exchanger (NCX) currents ($\int I_{NCX}$) and $[Ca^{2+}]_i$ ($\Delta F/F_O$) in response to rapid perfusion with 20 mmol/L caffeine, as performed previously.[27] Both $\int I_{NCX}$ and $[Ca^{2+}]_i$ were increased (P<0.001; n=6) in PDE3A$^{-/-}$ cardiomyocytes compared with WT (Figure 4 and Online Table II). Furthermore, Mil had no effect (P=0.284; n=6) on SR Ca^{2+} content in PDE3A$^{-/-}$ cardiomyocytes while increasing (P<0.05; n=8) SR Ca^{2+} content in WT (ie, from −0.71±0.07 to −0.96±0.07 pA/pF) to the same levels (P=0.49; n=6) measured in PDE3A$^{-/-}$ cells.

To identify the potential downstream molecular targets mediating the cAMP-dependent changes in Ca^{2+} homeostasis of myocardium from PDE3A$^{-/-}$ mice, we measured PLN and SR Ca^{2+} release channel (cardiac ryanodine receptor [RyR2]) phosphorylation levels. As seen in Figure 5, PLN phosphorylation at the PKA-dependent site, Ser-16, was elevated 2.1-fold (P<0.001; n=3) in PDE3A$^{-/-}$ myocardium compared with WT, without differences (P=0.940; Online Figure IV) in PLN phosphorylation at Thr-17, the Ca^{2+}-calmodulin kinase II–dependent site. RyR2 phosphorylation also was elevated (P<0.01) at both PKA-dependent site (Ser-2808 and Ser-2030) and the calmodulin kinase II–dependent site (Ser-2814) in PDE3A$^{-/-}$ myocardium compared with WT (Figure 6). PDE3A$^{-/-}$ hearts further showed elevated phosphorylation levels of several other PKA targets, including the cAMP response element-binding protein transcription factor, CREB, which could underlie the changes in SERCA2a and RyR2 expression seen in PDE3A$^{-/-}$ myocardium[28] (Online Figure V).

Because PKA-dependent modulation of SR Ca^{2+} uptake requires SERCA2a/PLN/AKAP18 molecular complexes,[14] we investigated whether PDE3A also might be part of this complex. Figure 7 demonstrates using immunoprecipitation studies that PDE3A colocalizes with SERCA2a, and Figure 8 demonstrates using immunoprecipitation studies that PDE3A interacts with SERCA2a, PLN, AKAP-18, PKA-RII, and protein phosphatase type 2A, but not with RyR2. The presence of PDE3A and SERCA2a in these immunoprecipitates was verified by liquid chromatography-tandem mass spectroscopy analysis (Online Figure VI). Interactions between these

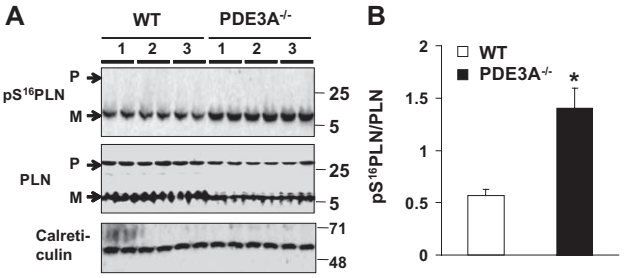

Figure 5. Increased levels of protein kinase A (PKA)–dependent phosphorylation of phospholamban in phosphodiesterase (PDE) 3A$^{-/-}$ hearts. Western blots of wild-type (WT) and PDE3$^{-/-}$ heart lysates (40 μg), illustrating the effect of PDE3A ablation on phosphorylation of phospholamban (PLN) at PKA-dependent site serine 16 (S^{16}). **A**, PDE3A ablation increased phosphorylation of PLN (pPLN), with respect to total PLN at residue serine 16. Calreticulin was used as indicator of equal loading conditions. **B**, Bar graph summarizing $pSer^{16}PLN/PLN_{total}$ ratios in WT and PDE3A$^{-/-}$ hearts; ≈2-fold increase in PDE3$^{-/-}$ lysates. *P<0.01 vs WT (n=3 independent experiments, each with duplicate samples from 3 WT and 3 PDE3$^{-/-}$ heart lysates). In some experiments, PLN was run on a SDS-PAGE gel in its monomeric (5 kDa) and pentameric forms (25 kDa). Total PLN was used for the analyses.

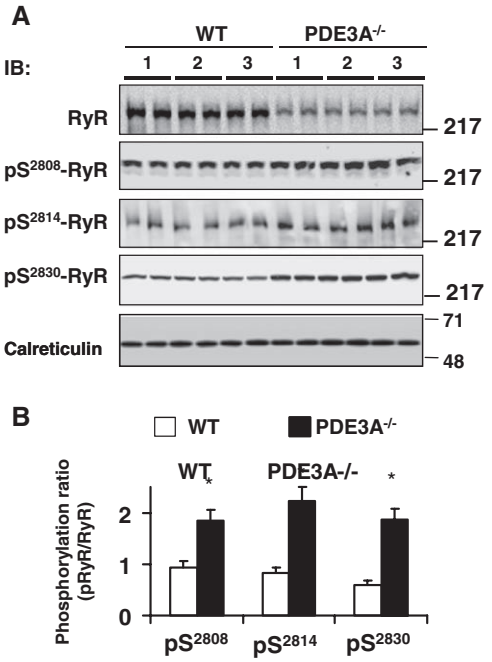

Figure 6. Increased levels of protein kinase A (PKA)– and calmodulin kinase II (CaMKII)–dependent phosphorylation of sarcoplasmic reticulum (SR) Ca^{2+} release channels in phosphodiesterase (PDE) $3A^{-/-}$ hearts. A, Western blots of wild-type (WT) and $PDE3^{-/-}$ heart lysates (50 μg/lane), illustrating the effect of PDE3A ablation on phosphorylation of SR Ca^{2+} release channel (cardiac ryanodine receptor [RyR2]) at PKA-dependent sites serine 2808 (pS^{2808}, Abcam 59225) and serine 2030 (pS^{230}, Badrilla A010-32), and CaMKII-dependent site serine 2814 (pS^{2814}, Badrilla A010-31). Calreticulin was used to confirm equal loading between the samples. **B**, Bar graph summarizing pRyR2/RyR2 ratios in WT and $PDE3A^{-/-}$ hearts; ≈2-fold increase in $PDE3^{-/-}$ lysates at all 3 sites.*P<0.01 vs WT (n=2 independent experiments, each with duplicate samples from 3 WT and 3 $PDE3^{-/-}$ heart lysates).

proteins also were detected using discontinuous sucrose gradient centrifugation studies of WT mouse cardiac membranes (Online Figure VII), which showed that PDE3A, SERCA2a, and PLN cofractionate.

Taken together, our findings support the conclusion that PDE3A regulates excitation–contraction coupling, Ca^{2+} transients, and contractility under basal conditions by modulating cAMP levels and PLN phosphorylation in SR microdomains containing the SERCA complexed with several of its regulatory partners. However, because CREB phosphorylation also is increased in $PDE3A^{-/-}$ hearts, it is conceivable that changes in the expression levels of Ca^{2+} handling proteins also might contribute to the functional alterations observed in $PDE3A^{-/-}$ mice. As summarized in Online Figure V, the expression of SERCA2a was increased in $PDE3A^{-/-}$ myocardium compared with WT, which was associated with increases (P<0.05) in both the maximal rate (V_{max}) of SERCA2a activity (56.8±3.4 nmol Pi/mg per minute) and the Ca^{2+} uptake rates (170.5±9 nmol Ca^{2+}/mg per 3 minutes) in SR vesicles isolated from $PDE3A^{-/-}$ myocardium compared with WT (33.05±4.5 nmol Pi/mg per minute and 81±3.7 nmol Ca^{2+}/mg per 3 minutes). Note that no difference (P=0.568) in Ca^{2+} sensitivity (K_m) of the SERCA2a activity was observed between the groups, which is expected when cAMP is absent[29] (Online Figure V). At first glance, SERCA2a elevations seem inconsistent with the observation that Ca^{2+} transients in the $PDE3A^{-/-}$ cardiomyocytes become indistinguishable from WT with Rp-cAMPS dialysis. However, RyR2 expression levels also were markedly reduced in the $PDE3A^{-/-}$ myocardium (Online Figure V), which is predicted to reduce SR Ca^{2+} release, despite the elevated SR Ca^{2+} load, in $PDE3A^{-/-}$ cardiomyocytes.

Discussion

It has long been established that PDE3 plays an important role in regulating intracellular cAMP levels and myocardial contractility in human hearts.[6,10,16] Accordingly, PDE3 inhibitors have been assessed in clinical trials for the treatment of heart failure. Unfortunately, chronic inhibition of PDE3 increases the incidence of ventricular arrhythmias and mortality.[15] Consequently, PDE3 inhibitors are only used to provide short-term inotropic support in conjunction with β-adrenergic blockers[30,31] in acutely decompensating cardiac patients. Although some previous studies have suggested that PDE3A is the predominant PDE3 isozyme expressed in the myocardium[32] and is important in regulating myocardial function,[10,16,33] PDE3B also has been reported to be a major regulator of murine myocardial contractility via its association with phosphatidylinositide 3-kinasesγ.[19]

Our studies establish that PDE3A is the isoform responsible for mediating the positive inotropic effects associated with the acute inhibition of PDE3s. Specifically, the loss of PDE3A (but not PDE3B) increases baseline myocardial contractility and eliminates the positive iontropic effects of Mil in isolated Langendorff hearts. These effects of PDE3A ablation on cardiac contractility were associated with cAMP-dependent elevations of Ca^{2+} transients without affecting L-type Ca^{2+} channels, thereby leading to enhancements in the excitation–contraction coupling gain. Although many factors could contribute to elevated Ca^{2+} cycling after PDE3A ablation, we found that $PDE3A^{-/-}$ myocardium had increased SR Ca^{2+} loading (measured via Ca^{2+} transients and integrated I_{NCX} in response to caffeine), which is a major regulator of excitation-contraction coupling gain.[26] Furthermore, Mil had no effect on $PDE3A^{-/-}$ myocytes but elevated Ca^{2+} transients and SR Ca^{2+} load (but not $I_{Ca,L}$) in WT myocytes, supporting further the conclusion that PDE3A underlies the PDE-dependent changes in baseline cardiac contractility, Ca^{2+} transients, and SR Ca^{2+} load.

The increased Ca^{2+} transients and SR Ca^{2+} loading in the $PDE3A^{-/-}$ myocardium occurred in association with enhanced SERCA activity (increased V_{max}) and Ca^{2+}uptake rates, elevated PLN phosphorylation levels (at the PKA-dependent site, S-16), increased SERCA2a expression levels, elevated RyR2 phosphorylation,[34] and reduced RyR2 expression. The changes in PLN and RyR2 phosphorylation suggest, as expected from compartmentation of PDE activity,[35,36] that PDE3A regulates cAMP locally in subcellular SR regions of cardiomyocytes, thereby controlling both PLN and RyR2 phosphorylation. Our coimmunoprecipitation studies further demonstrate that PDE3A associates with SERCA2a and PLN, as well as with several other proteins previously reported to assemble into macromolecular complex with SERCA2a, including the PKA

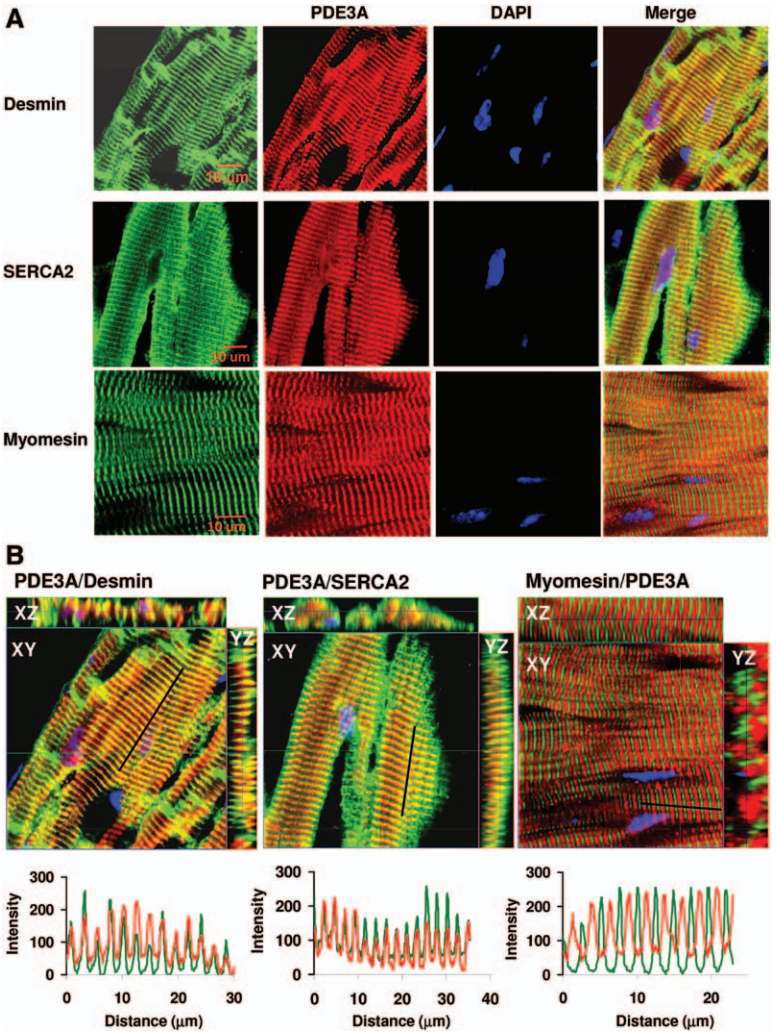

Figure 7. Phosphodiesterase (PDE) 3A belongs to the sarcoplasmic reticulum Ca2+ ATPase type 2a (SERCA2a)/phospholamban (PLN) macromolecular complex. A, As described in the Methods section, wild-type (WT) hearts (frozen sections) were incubated with rabbit anti–PDE3A-CT, anti-desmin, anti-SERCA2, and anti-myomesin primary antibodies, followed by AlexaFluor 488– or 594–conjugated anti-mouse or anti-rabbit secondary antibodies and imaged with Zeis LSM510 laser scanning confocal microscopy. Green stains for desmin (Z-lines) or SERCA2, whereas PDE3A is red and nuclei are blue (4',6-diamidino-2-phenylindole [DAPI]). Bar=10 μm. **B**, Merged images from stack of 6 to 8 sections (with 1-μm intervals) reveal colocalization of PDE3A with desmin and SERCA2 but not with myomesin (labeling M-line green). X–Y (**center**), above X–Z (**top**), and Y–Z (**right**) planes are at indicated positions.

regulatory subunit (PKA-RII), the AKAP (AKAP18 or AKAP 15/18δ), and protein phosphatase type 2A.[14] Remarkably, despite the increased RyR2 phosphorylation in PDE3A$^{-/-}$ myocardium, associations between PDE3A and RyR2 were not detected, suggesting that the regulation of cAMP levels by PDE3A at the levels of SERCA2a may have spillover effects in the vicinity of RyR2 channels.[24,37] Furthermore, we also have found in human myocardial SR fractions that PDE3 inhibition increased the effects of cAMP on Ca^{2+} uptake and that PDE3A coimmunoprecipitated with SERCA2a, PLN, AKAP18,PKA-RII, and protein phosphatase type 2A (Ahmad, unpublished observations, 2011). Collectively, these results support the conclusion that PDE3A regulates cAMP levels within a microdomain of the SR containing a macromolecular complex composed of SERCA2a and its major regulatory partners.[14]

Although the link between the changes in heart function and the cAMP-dependent increases in basal SR Ca^{2+}-ATPase activity induced by the loss of PDE3A is straightforward, the functional consequences of increased RyR2 phosphorylation and reduced RyR2 expression are less obvious. Single-channel studies have established that RyR2 phosphorylation by PKA (and by calmodulin II kinase) increases RyR2 channel open probability, which, in principle, could have competing effects on Ca^{2+} transients and contractility.[27,38] Specifically, increased RyR2 channel open probabilities not only can lead to enhanced SR Ca^{2+} transients but also causes elevated Ca^{2+} leak that can deplete SR Ca^{2+} stores, thereby reducing Ca^{2+} transients.[39] Because the loss of PDE3A was associated with elevated SR Ca^{2+} loads, it is reasonable to conclude that RyR2 phosphorylation did not lead to excessive Ca^{2+} leak, which is consistent with the absence of either increased Ca^{2+} sparks in PDE3A$^{-/-}$ cardiomyocytes or cardiac dysfunction in PDE3A$^{-/-}$ mice up to 6 months of age (data not shown). These observations support the conclusion that elevated RyR2 phosphorylation associated with PDE3A ablation contributes to the increased Ca^{2+} transients and cardiac contractility by enhancing RyR2 openings and SR Ca^{2+} release. Enhanced RyR2 openings also can help explain the rather modest acceleration of Ca^{2+} transient relaxation rates in PDE3A$^{-/-}$ cardiomyocytes, despite large elevations in SERCA2a activity. Specifically, enhanced RyR2 opening could lead to prolonged SR Ca^{2+} release, thereby counteracting the enhanced relaxation expected with accelerated SR Ca^{2+} uptake by SERCA2a.[27] Interestingly, although the phosphorylation levels of RyR2 channels were increased, RyR2 protein expression was reduced in PDE3A$^{-/-}$ myocardium, which is expected to reduce SR Ca^{2+} release. These changes in RyR2 expression might represent a compensatory response to limit the extent of Ca^{2+} transient elevations, and these changes could be mediated by

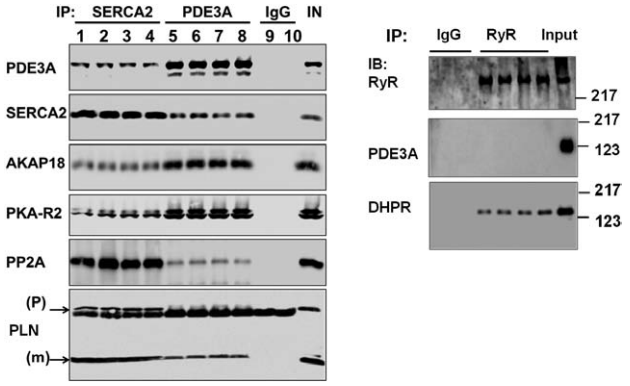

Figure 8. Phosphodiesterase (PDE) 3A coimmunoprecipitates with sarcoplasmic reticulum Ca^{2+} ATPase type 2a (SERCA2) in murine cardiac tissue. **A**, Representative Western blots from 2-way coimmunoprecipitation experiments illustrating that PDE3A interacts with SERCA2a, phospholamban (PLN), protein kinase A (PKA)-RII, and A-kinase anchoring protein (AKAP)-18. As described in the Methods section, precleared total wild-type (WT) heart lysates (1 mg) were incubated with anti-PDE3A (C terminal epitope) (3 μg) and anti-SERCA2a (3 μg) antibodies overnight at 4°C, followed by incubation with protein G beads (2 hours, 4°C). Immunoprecipitated proteins were eluted from the beads and subjected to SDS-PAGE/Western immunoblotting with the indicated antibodies. This experiment was representative of 3 independent experiments (1 heart/lane). **B**, Representative Western blots illustrating that PDE3A does not coimmunoprecipitate with the SR Ca^{2+} release channel (cardiac ryanodine receptor [RyR2]). Again, as described, precleared total heart WT lysates (1 mg) were incubated with anti-RyR antibodies, and immunoprecipitated proteins were subjected to SDS-PAGE/Western immunoblotting with the indicated antibodies. This experiment was representative of 2 independent experiments (1 heart/lane).

the increases in CREB phosphorylation. Clearly, more studies are warranted to more fully assess the consequences of altered RyR2 phosphorylation and RyR2 expression on Ca^{2+} homeostasis when PDE3 activity is inhibited. Examining the effects of PDE3 inhibition or PDE3A ablation in myocardium lacking either PLN or SERCA2a could be helpful in dissecting the relative consequences of PLN-SERCA2a vs other molecular targets in mediating the functional consequences of PDE3/PDE3A inhibition.

Previous clinical investigations have established that chronic treatment of heart failure patients with PDE3 inhibitors is associated with increased mortality. Consequently, these agents are contraindicated in the chronic treatment of cardiac patients.[15] Although the mechanism for the increased mortality seen in cardiac patients treated with PDE3 inhibitors is likely complex in model systems, chronic inhibition of PDE3 activity induces sustained elevations in the expression of the transcription repressor inducible cAMP early repressor, which is linked to increased cardiomyocyte apoptosis, via an autoregulatory positive-feedback loop.[40,41] These studies suggest that PDE3A$^{-/-}$ hearts might show long-term deterioration of cardiac function. However, despite evidence of the increased CREB phosphorylation typical of angiotensin II–induced and isoproterenol-induced heart failure, both hearts and isolated cardiomyocytes from PDE3A$^{-/-}$ mice showed enhanced cardiac function. Thus, it would appear that depletion of PDE3A alone is insufficient to induce cardiac dysfunction in mice under normal physiological conditions, and additional cardiac stresses or further aging is necessary to observe the detrimental effects of the loss of PDE3A function.

In PDE3A$^{-/-}$ mice, the absence of effects of Mil on heart and myocyte function, at doses that are expected to selectively inhibit both PDE3A and PDE3B activities,[22] establishes that PDE3B plays a minor role in the regulation of basal heart function. Although these observations are consistent with previous studies concluding that PDE3B activity in the heart is associated with nonmyocardial cells, such as vascular smooth muscle cells, fibroblasts, adipocytes, and blood cells,[32,33,42] another study suggested that PDE3B is present in cardiomyocytes, where it regulates myocardial cAMP levels and serves to acutely protect the heart after biomechanical stress.[19] If PDE3B activity is important primarily under conditions of cardiac stress, then the toxicity observed in the failed clinical trials using Mil might be related predominantly to PDE3B inhibition, making it plausible that a selective PDE3A inhibitor might be a useful strategy for providing positive inotropic activity to heart failure patients. Clearly, more studies will be required to dissect the relative roles of PDE3A vs PDE3B in normal and diseased myocardium.

Our results demonstrate that the loss of PDE3A leads to adaptive changes in the myocardial protein expression levels (SERCA2a, RyR2), possibly via CREB activation. However, these changes in expression do not fully explain the functional changes observed between PDE3A$^{-/-}$ and WT hearts. Pressure, Ca^{2+} transients, and SR Ca^{2+} levels in the myocardium of WT mice were elevated by PDE3 inhibitors to the levels seen in PDE3A$^{-/-}$ mice, whereas these parameters were only affected by inhibition of PKA with Rp-cAMPS in cardiomyocytes from PDE3A$^{-/-}$ mice. It is conceivable that these differences between the groups could be related to compensatory changes in the expression or activity of other PDE isozymes.[43] However, we found that the activity of PDE4, the other major murine cardiac PDE isozyme,[24,37,44] which also regulates Ca^{2+} transients and myocardial contractility,[9] was unaffected by PDE3A ablation.

In summary, we demonstrated that PDE3A is the major PDE3 isozyme involved in the regulation of baseline myocardial contractile function by modulating PKA-dependent PLN phosphorylation, and thus both SR Ca^{2+} loads and Ca^{2+} transients, without affecting L-type Ca^{2+} currents. This regulation by PDE3A occurs via selective regulation of cAMP levels in SR microdomains containing macromolecular complexes comprising PDE3A plus other constituents of the SERCA complex (including SERCA2a, PLN, PKA, protein phosphatase type 2A, and AKAP18). Because PDE3B may be essential and protective in response to cardiac stress,[19] it is conceivable that selective inhibition of PDE3A might be a useful strategy for reversing the reduced contractile function observed in heart disease, although their use may be limited by proarrhythmic effects associated with RyR2 phosphorylation.[38,39]

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Phosphodiesterases (PDEs) catalyze the breakdown of cAMP and thereby are critical regulators of cardiac function.
- Many different PDE isozymes are present in heart, and their individual function remains unclear.

What New Information Does This Article Contribute?

- PDE3A, but not PE3B, regulates baseline myocardial contractility by controlling the level of phospholamban phosphorylation and Ca^{2+} ATPase activity in microdomains of the sarcoplasmic reticulum (SR).

PDEs are critical determinants of cAMP-dependent signaling in myocardium. The role of different PDE isozymes, particularly PDE3A vs PDE3B, in regulating heart function remains unknown. We found that hearts from mice lacking PDE3A ($PDE3A^{-/-}$), but not PDE3B, have elevated cardiac contractility and were unresponsive to pharmacological PDE3 inhibition. The enhanced contractility in $PDE3A^{-/-}$ hearts was associated with cAMP-dependent elevations in Ca^{2+} transient amplitudes and SR Ca^{2+} content, without changes in L-type Ca^{2+} current ($I_{Ca,L}$). The loss of PDE3A eliminated >85% of the PDE3 activity and increased phospholamban phosphorylation and SR Ca^{2+} ATPase type 2a activity, as well as cardiac ryanodine receptor phosphorylation. PDE3A was found to associate with SR Ca^{2+} ATPase type 2a, phospholamban, A-kinase anchoring proteins-18, protein kinase A-RII, and protein phosphatase type 2A in a macromolecular complex. These findings establish that PDE3A is the primary PDE3 isozyme modulating basal cardiac contractility and SR Ca^{2+} content by regulating cAMP in microdomains containing macromolecular complexes of SR Ca^{2+} ATPase type 2a–phospholamban–PDE3A. Because PDE3B may protect against cardiac stress, selective inhibition of PDE3A might be a useful therapeutic strategy for correcting the impaired contractility observed in heart disease, although this approach may be limited by proarrhythmic effects associated with cardiac ryanodine receptor phosphorylation.