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Phosphodiesterase 4D (PDE4D) regulates baseline sarcoplasmic reticulum Ca^{2+} release and cardiac contractility, independently of L-type Ca^{2+}current

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Abstract

Rationale—Baseline contractility of mouse hearts is modulated in a PI3Kγ-dependent manner by type 4 phosphodiesterases (PDE4), which regulate cAMP levels within microdomains containing the sarcoplasmic reticular (SR) calcium-ATPase (SERCA2a).

Objective—To determine whether PDE4D regulates basal cAMP levels, phospholamban (PLN) phosphorylation and SERCA2a activity in SR microdomains.

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Disclosures
None.

Methods & Results—We assessed myocardial function in PDE4D-deficient ($PDE4D^{-/-}$) and littermate wild-type (WT) mice at 10-12 weeks of age. Baseline cardiac contractility in $PDE4D^{-/-}$ mice was elevated *in vivo* and in Langendorff perfused hearts, while isolated $PDE4D^{-/-}$ cardiomyocytes showed increased Ca^{2+} transient amplitudes and SR Ca^{2+}content, but unchanged $I_{Ca(L)}$, compared to WT. The PKA inhibitor, R_p-cAMPS, lowered Ca^{2+} transient amplitudes and SR Ca^{2+} content in $PDE4D^{-/-}$ cardiomyocytes to WT levels. The PDE4 inhibitor rolipram (ROL) had no effect on cardiac contractility, Ca^{2+} transients or SR Ca^{2+} content in $PDE4D^{-/-}$ preparations but increased these parameters in WT hearts to levels indistinguishable from those in $PDE4D^{-/-}$. The functional changes in $PDE4D^{-/-}$ myocardium were associated with increased PLN phosphorylation (pPLN) but not RyR2 receptor phosphorylation. ROL increased pPLN in WT cardiomyocytes to levels indistinguishable from those in $PDE4D^{-/-}$ cardiomyocytes. In murine and failing human hearts, PDE4D co-immunoprecipitated with SERCA2a but not with RyR2.

Conclusions—PDE4D regulates basal cAMP levels in SR microdomains through its interactions with SERCA2a-PLN. Since Ca^{2+} transient amplitudes are reduced in failing human myocardium, these observations may have therapeutic implications for patients with heart failure.

Keywords

PDE4D; cAMP; cardiac function; excitation-contraction coupling

Introduction

Cyclic nucleotide phosphodiesterases (PDEs) hydrolyze and inactivate cAMP. The PDE super-family is comprised of eleven gene families. Since individual families can contain as many as four genes that can be processed to yield multiple transcripts, most cells express numerous PDEs. Recent studies have established that certain PDEs can selectively interact with other cellular proteins, assemble into specialized macromolecular complexes within discrete functional compartments, and allow precise spatio-temporal control of cellular cAMP-mediated, PKA-dependent signalling[1;2]. The heart can express PDEs from each of the PDE1 through PDE5 families, as well as the PDE8 family[3]. Since reductions in cAMP/PKA-signalling contribute to impaired cardiac function in heart disease patients[4], PDE inhibitors were initially promoted for treating heart failure. However, despite their benefit in treating contractile failure, prolonged treatment of heart failure patients with PDE inhibitors increases mortality, principally by increasing sudden cardiac death[5]. Selective targeting of PDE isoenzymes may provide novel opportunities to correct the impaired cAMP-dependent signalling seen in heart disease without these adverse consequences.

Consistent with current paradigms of cAMP compartmentalization, PDE3 and PDE4 enzymes suppress basal cAMP/PKA-signalling and contractility in cellular microdomains containing sarcoplasmic reticulum (SR) Ca^{2+}pumps, but not ryanodine receptors (RyR2s) or L-type Ca^{2+}channels[6;7]. Since PDE4D isoforms associate with SERCA2a[7] and RyR2 receptors[8], we investigated PDE4D's role in regulating cardiac contractility. Mice lacking PDE4D have enhanced baseline cardiac contractility associated with increased PLN phosphorylation, SR Ca^{2+} content and Ca^{2+} transients but not elevated $I_{Ca,L}$ or RyR2

phosphorylation. PDE4D also co-assembles with SERCA2a but not with RyR2 in both murine and human hearts.

Methods

PDE4D deficient (PDE4D$^{-/-}$)[9] and littermate wild-type (WT) mice (129vj/c57 background) were studied at 10-12 weeks of age. Experiments were conducted in accordance with the Canadian Council of Animal Care. Detailed methods are in the Online Data Supplement at http://circres.ahajournals.org.

Results

Consistent with previous studies[8], PDE4D ablation did not affect heart-weight to bodyweight ratios or heart morphometry assessed with echocardiography (Online Table I) but did cause mild reductions ($P<0.005$) in mean arterial blood pressure (MAP) and elevations ($P<0.05$) in ventricular contractility compared to WT (Online Figure I & Table I). Since assessment of contractility is complicated by MAP differences, we studied Langendorff hearts, which showed elevated ($P<0.01$) left ventricular developed pressures (LVDP) as well as the peak time-derivatives of pressure (dP/dt_{max} and dP/dt_{min}) in PDE4D$^{-/-}$ hearts (Online Figure II, Online Table II). As expected[7], the PDE4 inhibitor rolipram (ROL) increased contractility and elevated heart rates (HR) in WT hearts; to eliminate the effect of the latter on contractility, hearts were paced at 9Hz, faster than the beating rate of ROL-treated hearts. Contractility (LVDP, dP/dt_{max} and dP/dt_{min}) remained higher ($P< 0.01$) in paced PDE4D$^{-/-}$ hearts compared to WT (Figure 1, Online Table II). ROL had no effect on PDE4D$^{-/-}$ hearts, but increased ($P<0.01$) contractility in WT to levels indistinguishable ($P= 0.14$) from PDE4D$^{-/-}$ (Online Table II). Shortening of isolated PDE4D$^{-/-}$ cardiomyocytes was also higher ($P<0.05$) and ROL augmented shortening ($P<0.05$) in WT cardiomyocytes to levels indistinguishable ($P=0.76$) from PDE4D$^{-/-}$ (Online Figure III). Thus, PDE4D ablation increases baseline ventricular myocardial contractility and eliminates inotropic responses to PDE4 inhibition.

To explore the cellular mechanisms underlying these PDE4D effects, Ca^{2+} transients and L-type Ca^{2+} currents ($I_{Ca,L}$) were simultaneously recorded in voltage-clamped ventricular cardiomyocytes. PDE4D$^{-/-}$ myocytes had increased ($P<0.01$) Ca^{2+} transient amplitudes and decay rates, without alterations in $I_{Ca,L}$ compared to WT (Figure 2, Online Table III). Intracellular dialysis with the PKA inhibitor R_p -cAMPS[10] had no effect on WT myocytes but reduced Ca^{2+} transients in PDE4D$^{-/-}$ to WT levels (Figure 2, Online Table III). On the other hand, the SR Ca^{2+} content (i.e. integrated Na^+/Ca^{2+} exchanger (NCX) currents following caffeine exposure) was higher ($P<0.01$) in PDE4D$^{-/-}$ myocytes than in WT myocytes, and R_p-cAMPS abolished these differences. ROL had no effect ($P=0.91$) on SR Ca^{2+} content of PDE4D$^{-/-}$ myocytes, but caused increases ($P<0.01$) in WT myocytes to levels indistinguishable ($P=0.86$) from those of PDE4D$^{-/-}$ myocytes (Figure 3).

Compared to WT, PDE4D$^{-/-}$ myocardium had elevated ($P<0.05$) PLN phosphorylation (pPLN) levels (Figure 3) which were indistinguishable from ROL-treated WT myocardium as well as either ROL-treated or ROL-untreated PDE4D$^{-/-}$ myocardium (not shown). In

contrast, RyR2 phosphorylation (Figure 3) was not different (P=0.11) at Serine 2030, the PKA-dependent site regulating function[11], or S2814 (P=0.79), but was unexpectedly[8] decreased (8%, P=0.01) at S2808. Co-immunoprecipitation in preparations from mouse and human myocardium revealed that PDE4D associates with SERCA2a but not RyR2 (Figure 4). Although PI3Kγ is required for PDE4 activity in micro-domains containing SERCA2a[8], it was not detected in SERCA2a immunoprecipitates, suggesting that PI3Kγ's enzymatic activity[6;7;12] is required. This is consistent with elevated contractility seen in Langendorff hearts treated with the PI3Kinase inhibitor, wortmannin (Online Figure VI).

Discussion

PDE4D$^{-/-}$ mice were hypotensive and had enhanced myocardial contractility associated with increases in cardiomyocyte shortening, Ca^{2+} transient amplitudes, Ca^{2+} transient relaxation rates and SR Ca^{2+} loads, without changes in $I_{Ca,L}$, compared to WT. PKA-inhibition with cAMP antagonists eliminated these cellular differences. In addition, PLN phosphorylation (at PKA site S-16), but not RyR2 phosphorylation, was elevated in PDE4D$^{-/-}$ hearts. As shown previously[7], PDE4D isoforms with molecular weights of ~97kDa (i.e. PDE4D3/8/9) co-immunoprecipitated with SERCA2a in both human and murine hearts. These findings support the conclusion that PDE4D regulates basal cAMP levels (and thus PKA activity) in macromolecular complexes containing the SR Ca^{2+}ATPase without functionally influencing (spatially adjacent) RyR2 receptors or L-type Ca^{2+}channels. Our inability to identify PI3Kγ in SERCA2 immunoprecipitates, suggests that PI3Kγ does not regulate PDE4D via protein-protein interactions, as with PDE3B[13], but requires enzymatic activity, as in mice lacking PTEN-phosphatase[12]. Indeed, wortmannin increased ventricular contractility/ relaxation (Online Figure VI).

Selective inhibition of PDE4[14] elevated contractility, Ca^{2+} transients, Ca^{2+} SR loads and PLN phosphorylation in WT hearts, as reported[7], to levels seen in PDE4D$^{-/-}$ myocardium without affecting PDE4D$^{-/-}$. Thus, although PDE4A and PDE4B are expressed in mouse heart[15], PDE4D underlies the cAMP-dependent baseline contractile responses to PDE4 inhibitors. Whilst PDE4D ablation does not induce expression changes of other PDE isoforms in different organs[9], changes in activity/expression of other PDE isozymes might have occurred. In this regard, responses to PDE3 inhibitors (which also affect mouse baseline contractility[7;16]) were unaffected by PDE4D ablation (Online Figure V). Although inhibition of other PDE isozymes was not examined, the consequences of PDE4D ablation on cardiac function can be readily explained by the loss of PDE4D activity alone.

A previous study reported that PDE4D and RyR2 interact in murine cardiomyocytes and that PDE4D$^{-/-}$ mice develop both heart dysfunction and arrhythmias by 9 months of age resulting from RyR2 hyperphosphorylation at S-2808[8]. We found neither elevated RyR2 phosphorylation in PDE4D$^{-/-}$ hearts nor evidence of direct interactions between PDE4D and RyR2 in murine hearts. These differences may be related to differences in reagents used in the two studies or to age-dependent changes in cAMP regulation and/or heart function[17]. In this regard, we did observe declines in cardiac function of PDE4D$^{-/-}$ mice at 9 months[8], which helps explain why another study[15], focusing on β-adrenergic responses, only identified acceleration of Ca^{2+} transient relaxation in PDE4D$^{-/-}$ myocytes at 5-6 months,

accompanied by trends (non-significant) towards elevations of Ca^{2+} transients as well as myocyte shortening/relaxation (discussed further, Online Supplement).

Age-dependent deterioration of PDE4D$^{-/-}$ heart function is not inconsistent with human data showing that persistent cAMP-dependent stimulation with, for example, PDE3 inhibitors promotes disease progression, mortality and arrhythmias in heart disease patients, despite providing short-term benefit (by enhancing contractility) in transplant and heart failure patients[18]. On the other hand, clinical trials with PDE4 inhibitors did not identify cardiovascular side-effects (besides slight increases in atrial fibrillation)[19]. These differences between mouse and humans may arise from higher relative PDE4 activities in mouse (~35%) versus human (<10%) myocardium[20]. However, PDE activity is highly compartmentalized[1], making local (not global) activity most relevant functionally. Thus, PDE4D tethering to SERCA2a combined with the high PDE4 activity (~50% of total activity) observed in PLN immunoprecipitates from human myocardium[20] suggests that PDE4D fine-tunes the cAMP-dependent SR Ca^{2+}-ATPase activity in human hearts[20]. This observation is particularly relevant since heart disease/failure is invariably associated with impairment of cAMP/PKA-dependent SR Ca^{2+}-ATPase activity. Moreover, SERCA2a gene therapy[21] or beta-blocker treatment (which enhance SERCA2a expression[22]) improves heart function and longevity. Thus, our findings support the possibility that selective PDE4D inhibition could prove beneficial for treating heart disease by specifically elevating cAMP in SR micro-domains containing SERCA2a/PLN. Remarkably, there is a paucity of definitive data available on direct actions of PDE4 inhibitors on cardiac function in humans or in animal models. Clearly, more studies are required to fully determine the role of PDE4D and its inhibition in heart disease patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non-standard Abbreviations and Acronyms

AKAP 18	A-kinase anchoring protein-18
cAMP	adenosine-3', 5' cyclic monophosphate
Ca^{2+} transients	whole cell Ca^{2+} transients
dP/dt_{max}	first positive time derivative of the maximum pressure development
dP/dt_{min}	first negative time derivative of the maximum pressure development

EDP	end diastolic pressure
$I_{Ca(L)}$	L-type calcium current
LVP	left ventricular pressure
LVDP	left ventricular developed pressure
MAP	mean arterial pressure
PDE4D	phosphodiesterase type 4D
PI3Kγ	phosphoinositide 3-kinase gamma
PLN	phospholamban
pPLN	phosphorylated phospholamban
PKA	protein kinase A
PP1	protein phosphatase 1
ROL	rolipram, PDE4 selective inhibitor
R_p-cAMPS	R_p-adenosine-3', 5' cyclic monophosphorothioate
RyR2	cardiac ryanodine receptor
SERCA2a	sarcoplasmic reticulum calcium ATPase type 2a

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Novelty and significance

"What is known?"

- Cyclic nucleotide phosphodiesterases (PDEs) are a complex family of enzymes encoded by 23 distinct genes that degrade cAMP/cGMP.
- PDEs are typically found in macromolecular complexes allowing tight spatial and temporal control of cAMP-dependent signalling in cellular microdomains.
- Family-specific PDE inhibitors are used clinically for inotropic support in heart failure patients; however, their prolonged use increases mortality.

"What new information does this article contribute?"

- PDE4D is tethered to the sarcoplasmic reticular (SR) Ca^{2+} ATPase type 2a (SERCA2a) thereby suppressing baseline cAMP/PKA-dependent Ca^{2+} cycling

Although PDE inhibitors can improve cardiac function, their prolonged use is associated with heart-related morbidity and mortality. Given the diversity of PDE isozymes, precise targeting of selected PDE isoforms is predicted to enable pinpoint modulation cAMP-dependent signalling in cellular microdomains. Indeed, we show that genetic elimination of PDE4D (one of 4 genes in the PDE4 family) elevates cAMP levels in subcellular compartments containing the SR Ca^{2+} pump (i.e. SERCA2a/phospholamban), but not L-type Ca^{2+} channels or cardiac ryanodine receptors, leading to elevations in phospholamban phosphorylation, SR Ca^{2+} levels, Ca^{2+} transients and cardiac contractility. This data shows that, in principle, selective pharmacological targeting of PDE4D in SR microdomains allows precise pharmacological control of cardiac contractility, while potentially minimizing the side-effects associated with broader spectrum inhibition. These findings might have significant implications in developing treatment strategies for heart disease patients.

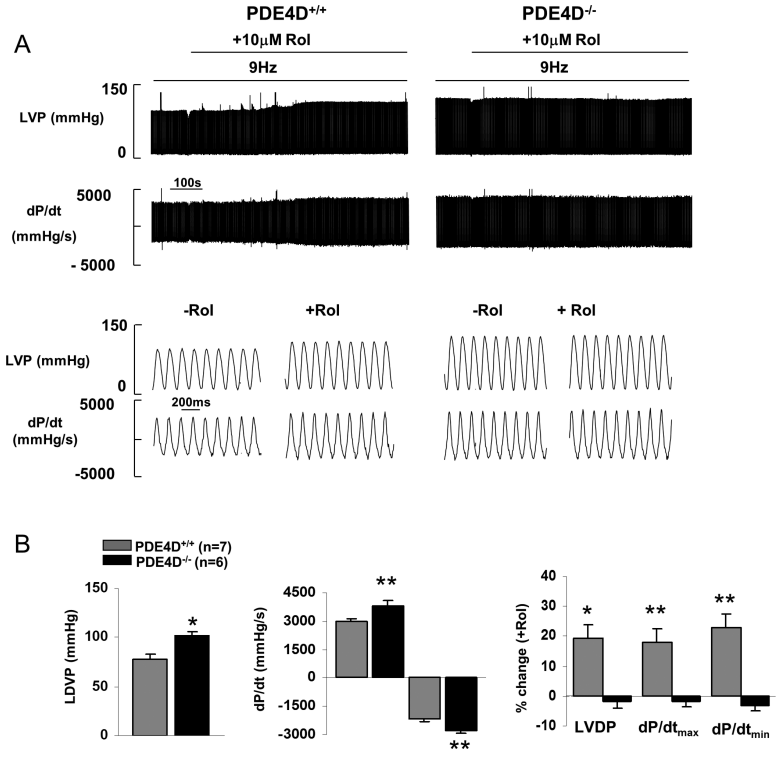

Figure 1. Assessment of Langendorff heart function

(A) Records of left ventricular pressure (top), dP/dt_{max} and dP/dt_{min} (bottom) measured in paced (9Hz) Langendorff-perfused hearts before and after ROL infusion. (B) Mean data for LVP (left), dP/dt_{max} and dP/dt_{min} (middle) and % change from baseline following ROL treatment (right), with baseline recorded after a 20 min equilibration period. *P<0.05 vs.WT, **P<0.01 vs. WT

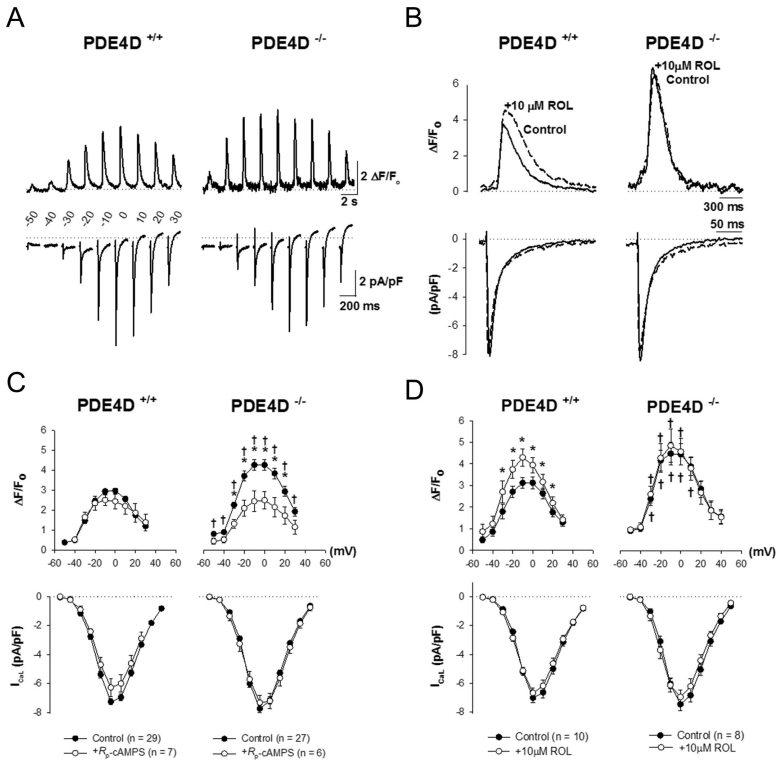

Figure 2. Ca^{2+} transients and I_{CaL} Measurements

(A) Ca^{2+} transients (upper) and I_{CaL} (lower) recorded for WT and PDE4D$^{-/-}$ cardiomyocytes in response to voltage steps (indicated) from –85mV holding potential and a 500 msec ramp to -45mV. (B) Typical Ca^{2+} transients and $I_{Ca,L}$ at +10mV before and after ROL application. Mean Ca^{2+} transient and $I_{Ca,L}$ peaks as a function of voltage in the presence and absence of RpcAMPS (C) and ROL (D). *P<0.01 versus control within same group; †P<0.01 versus WT control

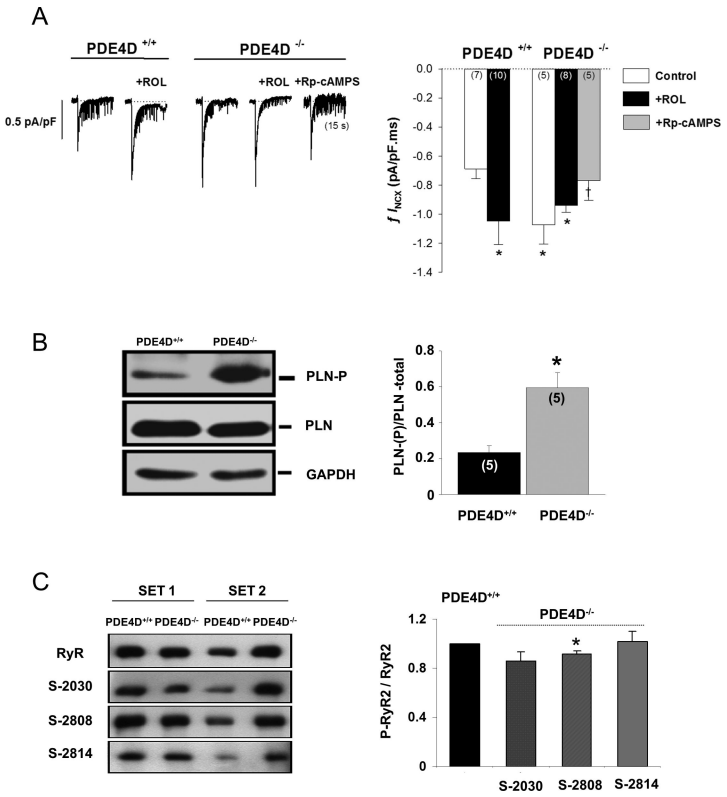

Figure 3. Measurements of SR Ca^{2+} content (A) as well as PLN and RyR2 phosphorylation (B&C)

(A) I_{NCX} evoked by a 10s application of 20mM caffeine in the presence or absence of RpcAMPS or ROL (*left*). Mean (time) integrated I_{NCX} to assess SR Ca^{2+} content bottom (*right*). *P< 0.05 versus WT. (B) Representative Western blot of protein extracts from left ventricular cardiomyocytes to measure phosphorylated PLN (*left*). Average intensity ratios of $pPLN/PLN_{total}$(*right*). *P< 0.05 versus WT; †P<0.05 vs. $PDE4D^{-/-}$ (n=5 hearts). (C) Representative WB of protein extracts from left ventricular cardiomyocytes illustrating the effect of PDE4D ablation on phosphorylated RyR2 (pRyR2) levels (*left*). Mean data showing changes in pRyR2/ $RyR2_{total}$ ratios in $PDE4D^{-/-}$ hearts (*right*). *P<0.05 versus PDE4D WT (n= 4 hearts).

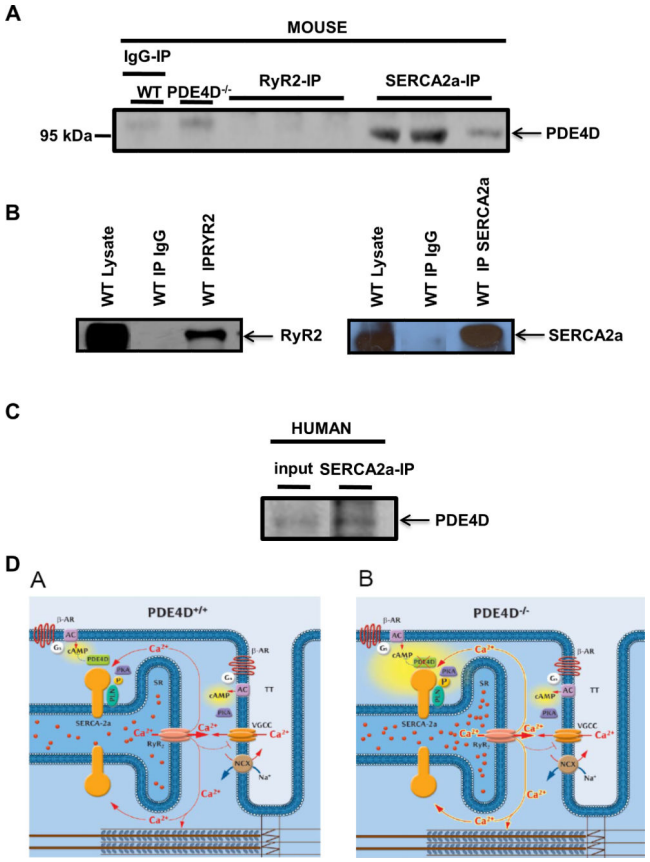

Figure 4. PDE4D interactions with SERCA2a in murine and human myocardium

A shows representative Western Blots (repeated in 3 separate hearts) probing with PDE4D in heart lysates from a PDE4D$^{-/-}$ mouse as well as for WT mouse heart homogenates that had been immunoprecipitated using control IgG, or using RyR2- or SERCA2a-specific antibodies. Results show that PDE4D antibodies recognized strong bands at MWs of 97kDaltons (corresponding to PDED-3,-7 and -9 splice variants of PDE4D) in immunoprecipitation reactions with anti-SERCA2a antibodies, but not with anti-RYR2 antibodies or with IgG controls. A very weak nonspecific band having MW ~ 110kDaltons was detected in all immunoprecipitation groups as well as in homogenates from the PDE4D-null mice, confirming this is a nonspecific band of unknown origin. B shows representative inputs for immunoprecipitation reactions shown in A. C shows results of SERCA2a immunoprecipitation in human hearts. (D) Diagram summarizing implications of our results in WT (left) and PDE4D$^{-/-}$ (right) myocardium.