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SYNERGIC PDE3 AND PDE4 CONTROL INTRACELLULAR cAMP AND CARDIAC EXCITATION-CONTRACTION COUPLING IN A PORCINE MODEL

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ABSTRACT

Aims

Cyclic AMP phosphodiesterases (PDEs) are important modulators of the cardiac response to β-adrenergic receptor (β-AR) stimulation. PDE3 is classically considered as the major cardiac PDE in large mammals and human, while PDE4 is preponderant in rodents. However, it remains unclear whether PDE4 also plays a functional role in large mammals. Our purpose was to understand the role of PDE4 in cAMP hydrolysis and excitation-contraction coupling (ECC) in the pig heart, a relevant pre-clinical model.

Methods and Results

Real-time cAMP variations were measured in isolated adult pig right ventricular myocytes (APVMs) using a Förster resonance energy transfer (FRET) biosensor. ECC was investigated in APVMs loaded with Fura-2 and paced at 1 Hz allowing simultaneous measurement of intracellular Ca^{2+} and sarcomere shortening. The expression of the different PDE4 isoforms was assessed by Western blot in pig right ventricle and APVMs. Similarly to PDE3 inhibition with cilostamide (Cil), PDE4 inhibition with Ro 20-1724 (Ro) increased cAMP levels and inotropy under basal conditions. PDE4 inhibition enhanced the effects of the non-selective β-AR agonist isoprenaline (Iso) and the effects of Cil, and increased spontaneous diastolic Ca^{2+} waves (SCWs) in these conditions. PDE3A, PDE4A, PDE4B and PDE4D isoforms are expressed in pig ventricle.. In APVMs isolated from a porcine model of repaired tetralogy of Fallot which leads to right ventricular failure, PDE4 inhibition also exerts inotropic and pro-arrhythmic effects.

Conclusions

Our results show that PDE4 controls ECC in APVMs and suggest that PDE4 inhibitors exert inotropic and pro-arrhythmic effects upon PDE3 inhibition or β-AR stimulation in our pre-clinical model. Thus, PDE4 inhibitors should be used with caution in clinics as they may lead to arrhythmogenic events upon stress.

INTRODUCTION

The β-adrenergic receptor (β-AR) signaling pathway is the main route for cardiac stimulation upon stress. It allows cardiac adaptation to increase blood supply to muscles during exercise. The so called “fight or flight” response starts with β-AR stimulation by catecholamines, leading to G_s activation of adenylyl cyclases which catalyze the conversion of adenosine triphosphate (ATP) to the second messenger 3',5'-cyclic AMP (cAMP) and pyrophosphate. Subsequently, cAMP promotes protein kinase A (PKA) activity which in turn phosphorylates key proteins of the excitation-contraction coupling (ECC) such as L-type Ca^{2+} channels ($Ca_V1.2$), ryanodine receptors (RYR2), phospholamban (PLB) and contractile proteins like troponin I and myosin-binding protein C (MyBP-C).[1] These events underlie the classical positive inotropic and lusitropic effects of acute β-AR stimulation.

The levels of cAMP are not only determined by synthesis, but are also finely tuned by degradation enzymes called cyclic nucleotide phosphodiesterases (PDEs).[2-4] PDEs are subdivided into 11 families, among which five hydrolyse cAMP in the heart: PDE1, which is activated by Ca^{2+}/calmodulin; PDE2, which is stimulated by cGMP; PDE3, which is inhibited by cGMP; PDE4 and PDE8. While PDE1 and PDE2 can hydrolyse both cAMP and cGMP, PDE3 preferentially hydrolyses cAMP and both PDE4 and PDE8A are specific for cAMP.[3, 5] In the myocardium which species??, the PDE3 and PDE4 families prevail to degrade cAMP and regulate ECC. PDE3 predominates in other large mammals[6] and in human.[7] PDE3 inhibition was once privileged as a therapeutic strategy to boost the weakening pump in heart failure (HF) where the β-AR cascade is desensitized.[8] However, although the clinically used PDE3 inhibitors milrinone and enoximone improve systolic function and alleviate the symptoms in acute HF,[9] their chronic use increases mortality, presumably by favoring cardiac arrhythmias.[10] Despite the fact that these drugs are widely presented as selective inhibitors of PDE3, milrinone and enoximone also inhibit PDE4 with similar potency.[11] This raises the intriguing possibility that PDE4 inhibition might contribute to both inotropic and pro-arrhythmic effects of PDE3 inhibitors in HF.

Numerous studies performed in rodents demonstrated the predominance of PDE4 for the control of cAMP signals generated by β-ARs,[12-14] of PKA phosphorylation of ECC proteins, and of Ca^{2+} homeostasis and contraction.[15, 16] Pharmacological inhibition of PDE4 was shown to enhance the pro-arrhythmic effect of β-AR stimulation in rat[16] and mouse ventricular cardiomyocytes.[17] The PDE4 family consists of four genes (*Pde4a-d*) but only *Pde4a*, *Pde4b*, and *Pde4d* appear to be expressed in rodents’ heart.[18, 19] In mice, genetic ablation of *Pde4b*

or *Pde4d* enhances the susceptibility to stress-induced ventricular tachycardia.[17, 19] This was attributed to hyperphosphorylation of RyR2 by PKA in *Pde4d*-deficient mice[17] and to exacerbated β-AR stimulation of L-type Ca^{2+} current in *Pde4b*-deficient mice. [19] Hyperphosphorylation of PLB was also reported in the latter study, probably because PDE4D associates with the PLB-SERCA2A complex to control its phosphorylation.[20] However, the role of PDE4 in the heart of human or large mammals remains elusive and even controversial. PDE4 is expressed in the human heart, [7, 17, 21] but it constitutes only ≈10% of the total cAMP-PDE activity (*versus* 40-60% in rat and mouse). [7, 17, 22] This appears to be due to a much higher activity of other PDEs in human *versus* rodents. [7] While it was initially reported that PDE4 does not control the contractile responses to catecholamines in atria from non-failing patients[23] or ventricular trabeculae from HF patients,[24] others showed redundancy of PDE3 and PDE4 to control the positive inotropic effects of serotonin in failing human hearts[25] and we demonstrated that these enzymes control β-AR responses and arrhythmias in human atria. [21] Similarly, we found in dog ventricular myocytes that PDE4 controls cAMP levels upon β-AR stimulation and modulate β-AR stimulation of the L-type Ca^{2+} current when PDE3 is inhibited.[26]Pig constitutes another classical pre-clinical model that exhibits gross anatomic structure very similar to that of humans and have been the subject of translational studies.[27] It closely resembles human cardiac physiology and HF pathophysiology is very similar to that of humans, thus it is widely used to study new therapeutic targets. PDE4 is expressed in the pig heart.[28] Jointly with PDE3, it controls basal cAMP levels and modulates the response to serotonin in pig atria.[29] It is also critical to control atrial inotropic and cAMP responses to β_1-AR stimulation in newborn piglets.[30] Surprisingly, unlike what was found at the atrial level, PDE3 and PDE4 were reported as minor to control ventricular responses to catecholamines in newborn piglets and only PDE3 inhibition increased the inotropic effect of β_2-AR stimulation[30] and of serotonin 5-HT_4 receptors stimulation[29] Nonetheless, in open-chest pigs, intramyocardial infusion of rolipram, a PDE4 inhibitor, induced ventricular tachycardia suggesting a role of this enzyme to control cAMP levels.[31] Furthermore, in adolescent animals, both PDE3 and PDE4 control ventricular responses to 5-HT.[29] suggesting age-dependent changes of relative activities. Therefore, the respective role of PDE3 and PDE4 in the adult pig heart, especially upon β-AR stimulation, remains elusive.This study was thus designed to characterize the functional role of PDE4 in this classical pre-clinical model.

We isolated ventricular myocytes from adult pig hearts and measured cAMP levels, using a Förster resonance energy transfer (FRET)-based sensor; Ca^{2+} transients (CaT) and sarcomere shortening (SS). Our study demonstrates that PDE4, along with PDE3, controls basal cAMP

levels and inotropic responses to β-AR stimulation. We also show that like PDE3, PDE4 limits ventricular arrhythmias by controlling Ca^{2+} homeostasis in normal adult pig right ventricular myocytes (APVMs) and in APVMs isolated from a model with right ventricular dysfunction reproducing repaired tetralogy of Fallot (rTOF).[32, 33] Like in rodents' heart, PDE3A, PDE4A, PDE4B and PDE4D isoforms are expressed in pig. Thus, our study suggests that many findings obtained in rodents concerning the role of PDE4 to control cardiac function might be transposable to pre-clinical model.

METHODS

All experiments were carried out according to the European Community guiding principles in the care and use of animals (2010/63/UE, 22 September 2010), the local Ethics committee (CEEA26 CAPSud) guidelines and the French decree n°2013-118, 1st February 2013 on the protection of animals used for scientific purposes (JORF n°0032, 7 February 2013 p2199, text n°24). Animal experiments were approved by the French Ministry of Agriculture (agreements N°14-027 and N°2016-125-7914). A surgical procedure mimicking repaired Tetralogy of Fallot (rTOF) to obtain right ventricular dysfunction secondary to chronic overload[32] was performed on 7 Landrace piglets (operated group) that were between 50 to 67 days old. 12 age-matched animals were used as healthy controls.. All animals were male to avoid bias related to hormonal variations. Echocardiographic assessment of RV function was performed before euthanasia. After completion of the study, animals were euthanized using lethal propofol infusion and exsanguination. (For more details, please see supplemental material).

Reagents

Isoproterenol from Sigma-Aldrich (Saint-Quentin, France) was freshly prepared in a 1 mg/mL ascorbic acid solution at 10 mM (Sigma-Aldrich, Saint-Quentin, France). Cilostamide (Cil) was from Tocris Bioscience (Bristol, UK): it blocks PDE3 with an IC50 ranging from 5 nM[34] to 27 nM,[35] and was used here at a 1 µM concentration. Ro 20-1724 (Ro, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidone) was from Calbiochem (Darmstadt, Germany): it blocks PDE4 with an IC50 value around 1 µM[36] and was used here at 10 µM. At these concentrations, Cil and Ro were shown to be selective for PDE3 and PDE4 respectively.

Myocyte isolation procedure

Hearts were excised from adult (5 to 7 months old) pigs and ventricular myocytes were enzymatically isolated from the right ventricular (RV) free wall as previously described. [32,

33]Briefly, the right coronary artery ostium was cannulated and the tissue was perfused with a constant flow of approximately 200 ml/min; temperature was maintained at 37°C. After 10 min washing with a Ca^{2+}-free Krebs-Ringer solution, tissue digestion was made by adding 0.354 UI/ml of collagenase A (Roche Diagnostic). After 15-20 minutes of enzymatic perfusion, the RV was removed and myocytes from endocardia and myocardial layers were mechanically collected, filtered, washed with a buffer solution (HEPES-BSA 2%), and resuspended in this buffer containing increasing Ca^{2+} concentrations up to 1.2 mM. Finally, isolated myocytes were plated on laminin-coated glass-bottom-dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) for 1h and maintained at 37°C. After 1h, the medium was replaced by 300 µL of FCS-free MEM or transduced with an adenovirus encoding the Epac-S^{H187} FRET-based sensor[37] at a multiplicity of infection (MOI) of 1000 pfu/cell.

Measurement of sarcomere shortening and Ca^{2+} transient

All experiments were performed at 30 ± 2°C. Freshly isolated APVMs were loaded with 1 µmol/L Fura-2 AM (Invitrogen) for 15 min in a Ringer solution containing (in mM): NaCl 121.6; KCl 5.4; Na-pyruvate 5; $NaHCO_3$ 4.013; NaH_2PO_4 0.8; $CaCl_2$ 1.8; $MgCl_2$ 1.8; glucose 5 and HEPES 10 (pH 7.4 with NaOH). Sarcomere shortening and Fura-2 ratio (measured at 512 nm upon excitation at 340 and 380 nm) were simultaneously recorded in Ringer solution, using a double excitation spectrofluorimeter coupled with a video detection system (IonOptix, Milton, MA, USA). Myocytes were electrically stimulated with biphasic field pulses (5 V, 4 ms) at a frequency of 1 Hz. Ca^{2+} transient amplitude was measured by dividing the twitch amplitude (difference between the peak systolic and the end-diastolic ratios) by the end-diastolic ratio, thus corresponding to the percentage of variation in the Fura-2 ratio. Similarly, sarcomere shortening was assessed by its percentage of variation, obtained by dividing the twitch amplitude (ΔL, difference between the end-diastolic and the peak systolic sarcomere length) by the end-diastolic sarcomere length (L_0). Relaxation kinetics were estimated by a non-linear fit of the decaying part of the Ca^{2+} transient and sarcomere shortening traces with the following equation: $Y(t)=A*\exp(-t/\tau)+A_0$, where t is the time, A_0 the asymptote of the exponential, A the relative amplitude of the exponential, and τ the time constant of the exponential.

FRET imaging

FRET experiments were performed at room temperature 24h after cell plating. Cells were maintained in a Ringer solution containing (in mM): NaCl 121.6, KCl 5.4, $MgCl_2$ 1.8; $CaCl_2$ 1.8; $NaHCO_3$ 4, NaH_2PO_4 0.8, D-glucose 5, sodium pyruvate 5, HEPES 10, adjusted to pH 7.4. Images were captured every 5 s using the 40x oil immersion objective of a Nikon TE 300

inverted microscope connected to a software-controlled (Metafluor, Molecular Devices, Sunnyvale, CA, USA) cooled charge coupled (CCD) camera (Sensicam PE, PCO, Kelheim, Germany). Cells were excited during 150-300 ms by a Xenon lamp (100 W, Nikon, Champigny-sur-Marne, France) using a 440/20BP filter and a 455LP dichroic mirror. Dual emission imaging was performed using an Optosplit II emission splitter (Cairn Research, Faversham, UK) equipped with a 495LP dichroic mirror and BP filters 470/30 (CFP) and 535/30 (YFP), respectively. Spectral bleed-through into the YFP channel was subtracted using the formula: YFP_{corr}=YFP-0.6xCFP.

Cell extracts and western blot analysis

RV tissue or isolated APVMs 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 and Complete Protease Inhibitor Tablets (Roche Diagnostics). Lysates were rotated at 4˚C for 30 min followed by a 10 min centrifugation at 20,000 x g and 4°C. Supernatants were directly used for Western blotting. 15 µg protein extracts were loaded. PDE3A was detected using a rabbit polyclonal anti-PDE3A antibody from Fabgenix. For specific PDE4A and PDE4B detection, rabbit polyclonal antibodies generated against their respective C-termini were used (anti-PDE4A: AC55; anti-PDE4B: 113-4). Mouse monoclonal antibody (ICOS PDE4D) was used to specifically detect PDE4D. PDE3A antibody was a generous gift from Dr Chen Yan (Rochester University, NY, USA). PDE4A, PDE4B and PDE4D antibodies were kindly provided by Pr Marco Conti (University of California San Francisco, CA, USA).

Statistics

All results are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism software (GraphPad software, Inc., La Jolla, CA, USA). Normal distribution was tested by a Shapiro-Wilk normality test. For normally distributed data, differences between multiple groups were analyzed using a nested ANOVA (which takes into account both the number of observations and the number of animals) was performed using the lmer function in the nlme v3.1–131 package for R (R version 3.4.1 and RStudio version 1.0.153), followed by Tukey's *post-hoc* test for all data obtained on individual cells. When the data obtained did not follow a normal distribution, a Kruskal-Wallis followed by a Dunn's *post hoc* test was used. To analyze results obtained with western blots, the mean values of two groups were analyzed by a Mann-Whitney test. A Chi^2 test followed by a Fischer exact test was used to compare number of arrhythmic cells. Differences with p-values <0.05 were considered as statistically significant. The

number of independent experiments performed and the statistical tests performed are indicated in the figures and their legends respectively.

RESULTS

Both PDE3 and PDE4 control cAMP levels in APVMs

To evaluate cAMP levels in isolated cardiomyocytes, the FRET-based sensor Epac-S^{H187} was expressed in APVMs using a recombinant adenovirus. As shown in Figure 1, continuous application of the non-selective β-AR agonist isoprenaline (Iso, 10 nM) increased the CFP/YFP ratio by 45.5 ± 4.8% (n=20, p<0.05) indicating an increased global cytosolic cAMP concentration. Addition of Ro 20-1724 (Ro, 10 µmol/L), a selective inhibitor of PDE4, increased the CFP/YFP ratio up to 184 ± 12.6% (n=20, p<0.001) demonstrating a major role for this enzyme to degrade cAMP produced upon β-AR stimulation. The selective PDE3 inhibitor cilostamide (Cil, 1 µmol/L) also increased the CFP/YFP ratio to similar levels (187.9 ± 15.3%, n=20, p<0.001). In the absence of β-AR stimulation, Ro or Cil alone induced a slight (<20%) but significant increase in basal CFP/YFP ratio (Figure 1B and D). However, concomitant inhibition of PDE3 and PDE4 resulted in a substantial cAMP elevation (+130.2 ± 13.9 %, n=12, p<0.001, Figure 1B and D). These results indicate that both PDE3 and PDE4 are important to counterbalance basal and β-AR-stimulated cAMP synthesis in APVMs.

PDE3 and PDE4 were reported to be decreased in pathological conditions such as hypertrophy and HF[17, 38-40] although this may depend on disease etiology and stage.[41-43] Thus, in a next series of experiments, we investigated the respective contribution of these enzymes in APVMs isolated from a pig model of right ventricle dysfunction induced by chronic overload as observed in humans with repaired tetralogy of Fallot (rTOF). [32, 33] As indicated in Supplemental Table 1, four months after pulmonary valve surgery and pulmonary artery banding, RV dimensions were largely increased, and Tricuspid Annular Plane Systolic Excursion (TAPSE) was decreased, attesting RV remodeling and dysfunction due to combined volume and pressure overload. Measurements of cAMP levels by FRET in APVMs from these animals showed no apparent effect of PDE3 and PDE4 inhibitors under basal conditions, whereas concomitant application of Ro and Cil was still able to increase cAMP (Supplemental Figure 1). The response to β-AR stimulation (Iso, 10 nM) was virtually absent in APVMs from the rTOF model, but cAMP could still be increased by concomitant application of PDE3 or PDE4 inhibitors..

Both PDE3 and PDE4 inhibition produce inotropic effects under basal conditions and promote pro-arrhythmogenic Ca^{2+} waves in APVMs

To investigate the role of PDE4 in ECC in APVMs, calcium transients (CaT) and sarcomere shortening (SS) were simultaneously recorded in cells loaded with 1 µmol/L Fura-2 and paced at 1 Hz (Figure 2A). Average diastolic sarcomere length was 1.79 ± 0.01 µm (n=13). Under control conditions (Ctrl), mean CaT amplitude was 35.5 ± 6.0% above diastolic Fura-2 ratio, and SS was 3.1 ± 0.9% of diastolic sarcomere length (n=13). CaT and SS declined to diastolic levels with average time constants (τ) of 0.42 ± 0.04 s and 0.15 ± 0.03 s, respectively (Figure 2B and C). Under basal conditions, Ro increased SS by about 3-fold (to 10.8 ± 3.1%, n=6, $p<0.01$) and increased CaT amplitude to 60.2 ± 11.4%, although this increase did not reach statistical significance (n=6). Similarly, inhibition of PDE3 by Cil potentiated both SS (8.7 ± 1.4 %, n=7, $p<0.01$) and CaT amplitude but not significantly (68.9 ± 12.1%, n=7, p=0.06). These inotropic effects were accompanied with a slight but not significant lusitropic effect (Figure 2C). Combination of both Ro and Cil produced a robust inotropic effect, increasing SS up to 13.7 ± 1.0% and systolic Ca^{2+} levels up to 72.0 ± 8.9% above diastolic ratio (n=13, $p<0.01$). Cil+Ro accelerated the return to diastolic Ca^{2+} by decreasing the τ value to 0.18 ± 0.03 s (n=13, $p<0.01$). However, the lusitropic effects of Cil, Ro or the combination of both inhibitors were not strong enough to reach statistical significance, suggesting that the low turnover of cAMP synthesis under basal conditions revealed by FRET imaging (Figure 1) could influence Ca^{2+} refilling of the SR but not contractile protein phosphorylation.

The crucial role for PDE4 and PDE3 to control cAMP levels and Ca^{2+} homeostasis was further demonstrated by the appearance of spontaneous Ca^{2+} waves (SCWs) upon cessation of pacing when PDE inhibitors were applied. When cells were subjected to Ro or Cil alone, very few SCWs were observed (Figure 3) but upon concomitant Ro and Cil perfusion, ~80% of the cardiomyocytes (n=14, $p<0.01$) exhibited spontaneous Ca^{2+} waves (SCWs) between two stimulations or during a 10 s pause of stimulation (2.3 ± 0.9 SCWs per 10 s, $p<0.01$). Because inhibition of both enzymes leads to pro-arrhythmic events, it demonstrates that both PDE4 and PDE3 contribute to Ca^{2+} homeostasis in APVMs. Interestingly, similar results were observed in APVMs isolated from rTOF animals. Whereas SS and CaT amplitude measured in APVMs isolated from healthy or rTOF animals were identical (Supplemental Table 2), relaxation and CaT decay were slower in rTOF (Supplemental Table 2), evoking a decreased velocity of SR Ca^{2+} uptake as we previously observed in this model.[33]

In rTOF APVMs, PDE4 or PDE3 inhibition had no significant inotropic or lusitropic effects under basal conditions, but concomitant application of these inhibitors nearly doubled CaT amplitude (n=12, p<0.01, Figure 4B), produced a 5-fold increase in SS amplitude (n=12, p<0.001, Figure 4C) and significantly accelerated CaT and SS relaxation (n=12, p<0.05, Figures 4B, 4C). Inhibition of both enzymes was also required to induce pro-arrhythmogenic SCWs in 50% of these cells (n=14, p<0.01, Figure 4E, 4F), demonstrating that not only in physiological but also under pathological conditions, both PDE4 and PDE3 control ECC and contribute to Ca^{2+} homeostasis in APVMs.

PDE4 and PDE3 modulate β-AR stimulation of ECC in APVMs

To investigate the functional consequences of PDE4 inhibition compared to PDE3 inhibition on β-AR-stimulated ECC in APVMs, cells were first subjected to a submaximal concentration of the non-selective β-AR agonist Iso (10 nM) and then to either inhibition of PDE4 with Ro or of PDE3 with Cil, as illustrated by the individual traces of CaT and SS in Figure 5A and 5B. As shown in Figure 5C, on average, Iso increased CaT amplitude from 19.3 ± 2.6% to 70.5 ± 7.1% (n=16, p<0.001 *vs* Ctrl), and SS was increased from 1.5 ± 0.3% to 11.9 ± 1.1% (n=16, p<0.001 *vs* Ctrl). Iso also strongly accelerated the relaxation rates of both parameters, with τ values decreasing from 0.4 ± 0.03 s to 0.26 ± 0.04 s for CaT (n=16, p<0.05 *vs* Ctrl) and from 0.24 ± 0.05 s to 0.04 ± 0.005 s for SS (n=15, p<0.001 *vs* Ctrl). These inotropic and lusitropic effects were potentiated by PDE4 inhibition. CaT was further increased to 78.5 ± 7.1% and SS to 15.0 ± 0.8% under Iso+Ro (n=16). Decay kinetics of CaT were also further accelerated by Ro (τ = 0.12 ± 0.01s, n=16, p<0.05 *vs* Iso, Figure 5C). This was also the case for SS relaxation, although the difference with Iso alone was modest because β-AR stimulation alone already accelerated drastically myocyte relaxation. PDE3 inhibition with Cil induced very similar effects as Ro (Figure 5D).

In these experiments, we also analyzed the occurrence of SCWs upon cessation of stimulation (Figure 6). Figure 6A and 6B illustrate representative recordings of CaT in normal Ringer, upon stimulation with Iso 10 nM alone and in combination with either Ro (Figure 6A) or Cil (Figure 6B). When cells were subjected to Iso alone, only sparse SCWs were observed (~1.5 per 10 s) in ~40% of cells. However, when Ro was applied in combination with Iso, 75% of the cells exhibited pro-arrhythmogenic SCWs at a frequency of 2.2 ± 0.5 per 10 s (Figure 6C). Again, these pro-arrhythmic effects were very similar to those observed upon PDE3 inhibition (Iso+Cil) which triggered SCWs at a frequency of 3.9 ± 1.6 per 10 s in 73.3% of cells (p<0.05 *vs* Iso) (Figure 6D).

PDE3A, PDE4A, PDE4B and PDE4D isoforms are expressed in pig right ventricle

PDE3A is a major PDE3 isoform degrading cAMP in human[44] and rodent[45] cardiomyocytes to control ECC coupling. PDE4 activity is due to the expression of PDE4A, PDE4B, and PDE4D variants in mouse[19] , rat[18, 40] and human[7] heart. To investigate whether these isoforms are also expressed in the porcine myocardium, we performed Western blot analysis using subtype-specific antibodies against PDE3A, PDE4A, PDE4B, and PDE4D. All four variants were detected in right ventricle homogenates (Figure 7A), indicating conservation between rodents, human and pig cardiac tissue. Importantly, the three PDE4 isoforms were also detected in protein extracts obtained from isolated cardiomyocytes (Figure 7B) demonstrating their expression in contractile cells.

DISCUSSION

Cyclic nucleotide phosphodiesterases are essential enzymes degrading cAMP not only to terminate β-AR stimulation of cardiac function, but also to compartmentalize cAMP signals within discrete domains inside cardiomyocytes.[3, 4] Literature is sparse and functional data are often missing in studies dedicated to the role of PDEs especially PDE4 in large mammals and human heart, . This is due to the limited access to human biopsies and the difficulty to isolate cardiomyocytes from explanted human ventricles. Compared to rodents, pig cardiac anatomy and physiology is much more similar to humans.[27] Therefore, it constitutes a good alternative model and a bridge to fill the gap between proof-of-concept studies performed in rodents and clinical trials in patients. Because genetic engineering of pigs is developing, its use as a preclinical model will rise.[46] However, despite a large amount of work realized in rodents that unveiled the preponderant role of PDE4 to control cardiac function, its participation in large mammals, especially in pig heart, remains elusive. Our study provides a unique panel of data describing for the first time at the cellular level, the respective role of PDE3 and PDE4 in this large mammal preclinical model.

Similar PDE4 isoforms are expressed in pig ventricular tissue than in rodents and human heart

We show here that the three PDE4 isoforms, PDE4A, PDE4B and PDE4D known to be expressed in rodents[19] and humans[17] are also expressed in pig ventricular tissue and

myocytes. This suggests that the expression profile of PDE4 isoforms is conserved in this preclinical model. In the rodent heart, PDE4 variants are localized in discrete microdomains, allowing fine tuning of cAMP signaling to control the phosphorylation and hence the activity of individual proteins such as β-ARs,[47] $Ca_v1.2$, [19] RyR2,[17] and PLB/SERCA2[20] within these compartments. PDE4 has also been found tethered to similar macromolecular signaling complexes in humans, including the RyR2, [17] PLB/SERCA2 and the β_1-AR complexes.[7] The inotropic and lusitropic effects of PDE4 inhibition reported here suggests that PDE4 might also control $Ca_V1.2$, RyR2, PLB phosphorylation by PKA and activity in APVMs. In rodents, different β-AR subtypes, namely β_1-AR and β_2-AR, mediate these effects under the control of both PDE3 and PDE4.[14, 15, 48] β_2-AR are localized within the t-tubules in rodent ventricular cells where cAMP is confined by PDE4,[49] and more specifically by PDE4B and PDE4D isoforms.[50] Interestingly, we show here that PDE4B and PDE4D isoforms are also expressed in pig ventriculocytes. Whether cAMP emanating from β_1-AR and β_2-AR within the t-tubules is confined by the same PDE4 isoforms in pigs will require further investigations. A more detailed comparison in terms of level of expression of various PDE families, association with the key proteins of the ECC and function is also required to determine whether this model fully recapitulates the role of PDEs in the rodent and human hearts.

Both PDE3 and PDE4 control cAMP and ECC in pig ventricular myocytes

It is widely recognized that PDE3 is the main PDE isozyme controlling ventricular contractility in large animal models, which are believed to exhibit a pattern of PDE expression close to human, where PDE3 dominates.[6, 21, 51] PDE3 being one of the main enzyme degrading cAMP in human heart, .[7] PDE3 inhibitors are potent cardiotonic agents with proven beneficial hemodynamic actions,[9] but their use is now limited to acute heart failure or post-surgery since chronic treatment promotes sudden cardiac death due to arrhythmias.[10] As expected, we confirm here that PDE3 inhibition increases cAMP levels under basal conditions and upon β-AR stimulation and exerts inotropic and lusitropic effects in APVMs. This is compatible with PDE3 being a major enzyme controlling cAMP levels in this species where, like in other large mammals such as bovine[52] and dog,[26, 51] [53]it is predominantly expressed. We also show that the PDE3A isoform is present in porcine ventricular tissue, like in human heart where it is the main isoform controlling PLB phosphorylation.[54] Similarly to PDE3 inhibition, PDE4 inhibition also increases basal cAMP levels, contraction and relaxation in APVMs. While these effects of either PDE3 or PDE4 inhibitors are relatively modest, their concomitant application has a drastic impact on cAMP levels and consequently amplifies calcium transient amplitude and

sarcomere shortening. This reveals that both PDE3 and PDE4 are redundant and concur to counterbalance cAMP synthesis under basal conditions as previously shown.[29] Upon β-AR stimulation, inhibition of either enzyme leads to an increase of cAMP levels potentiating ECC in APVMs, similarly to what was reported in ventricular cardiomyocytes isolated from rat,[14, 15] dog hearts[26] and from human atrial cells.[21] Our observations demonstrate the importance of the PDE4 family to control cardiac ECC in pig ventriculocytes like it has been described in rodent ventricular myocytes.[14-16] revealing that the role of this enzyme is conserved across species.
In accordance with the decreased PDE3 and PDE4 activities in a rat model of cardiac hypertrophy induced by chronic aortic constriction[40] and in HF patients,[17, 38] we show here that in a porcine model of right ventricular dysfunction secondary to chronic overload, mimicking the rTOF, [32, 33] PDE3 and PDE4 inhibitors are less effective to increase cAMP levels as previously reported in hypertrophied rat cardiomyocytes[40] and HF dogs. [38] This is also probably due to reduced cAMP synthesis as observed generally in HF[8] and suggested here by the reduced capacity of Iso to increase cAMP in APVMs from rTOF pigs (Supplemental Figure 1). This is probably this desensitization which might have hindered previous attempts to detect the effects of PDE4 inhibitors in human explanted biopsies from HF patients. [24] However, despite this desensitization, concomitant application of PDE3 and PDE4 inhibitors still resulted in significant increase in cAMP and in positive inotropic and lusitropic effects, suggesting that PDE4 and PDE3 control cardiac function not only in physiological but also in pathological conditions.

PDE4 inhibition is pro-arrhythmic in pig ventricular cardiomyocytes

Unlike the pro-arrhythmic effects of PDE3 inhibitors which are well documented and precluded their chronic use in HF,[10] the potential deleterious effects of PDE4 inhibitors on cardiac function in large mammals are scarce in the literature. Pro-arrhythmic effects of rolipram, a selective PDE4 inhibitor, have been observed in anesthetized open-chest adult pigs[31] and in isolated human atrium. [21] Our results here clearly demonstrate that PDE4 inhibition is pro-arrhythmic in APVMs but this requires prior elevation of cAMP with either PDE3 inhibition or β-AR stimulation, similarly to what we observed in rat ventricular cells.[16] Strikingly, the sole PDE3 inhibition produces only few arrhythmias and requires concomitant PDE4 inhibition to evoke SCWs in the majority of cells. Whether concomitant PDE4 and PDE3 inhibition is also required in human ventricular cells is therefore questionable. Indeed, it has been shown that milrinone and enoximone are not only PDE3 inhibitors but also showed similar potency to inhibit

PDE4. [11] Whether the deleterious effects of chronic PDE3 inhibition in patients were in fact due to concomitant PDE3 and PDE4 inhibition is thus suggested by the present study performed in an animal model closer to human than rodents. PDE4 inhibitors are new promising therapeutic agents and are currently developed to treat inflammation, chronic obstructive pulmonary disease, psoriasis, and neurological illnesses.[55] Our study also underlies that pre-clinical studies realized in large animals such as pig should carefully address the potential cardiac adverse effects of these new drugs especially under stress conditions, i.e. upon β-AR stimulation or combination with PDE3 inhibitors, when PDE4 inhibition has an impact on heart function.

CONCLUSION

Our results demonstrate that the previously reported conservation of the expression pattern of PDE4 isoforms among rodent and human hearts[7] is also applicable to pig ventricle. Our study demonstrates that PDE4 controls cAMP levels and ECC in healthy pigs and in a pathological model of RV overload. Therefore, it validates the pig as a relevant pre-clinical model to study the impact of PDE4 inhibitors on cardiac function under physiological and pathophysiological conditions. Importantly, it suggests some vigilance in the use of PDE4 inhibitors in clinic as they may lead to arrhythmogenic events.

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FIGURE LEGENDS

Figure 1. Effect of PDE4 or PDE3 inhibition on cAMP homeostasis in APVMs

(A, B) Time courses of the CFP/YFP ratio upon β-AR stimulation by Iso (10 nM) (A) or under basal conditions (B), after addition of the PDE3 inhibitor Cilostamide (Cil, 1 µM), the PDE4 inhibitor Ro 20-1724 (Ro, 10 µM) or combination of both inhibitors in APVMs expressing the cAMP sensor Epac-S^{H187}. Pseudo-color images of the CFP/YFP ratio were recorded at the times indicated by the letters on the graphs (A). **(C, D)** Mean variation (± SEM) of the normalized CFP/YFP ratio for each condition tested. Numbers indicate the number of cells from 3 pigs. Statistical significance is indicated as *, $p<0.05$, **, $p<0.01$; ***, $p<0.001$; a Kruskal-Wallis followed by a Dunn's *post hoc* test.

Figure 2. Effect of PDE3 or PDE4 inhibition on EC coupling in APVMs

(A) Representative traces of Ca^{2+} transients and sarcomere length variation ($\Delta L/L_0$) recorded in Fura-2-loaded APVMs paced at 1 Hz in basal conditions (Ctrl), after addition of the PDE4 inhibitor (Ro 20-1724, Ro, 10 µM) alone or in combination with the PDE3 inhibitor (Cilostamide, Cil, 1 µM). **(B)** Mean amplitude (±SEM) of Ca^{2+} transients and average relaxation kinetics Tau (± SEM) for each condition tested. **(C)** Sarcomere shortening and average relaxation kinetics Tau (± SEM) for all conditions tested. Numbers indicate the number of cells from 3 pigs. Statistical significance is indicated as: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; a nested ANOVA followed by Tukey's *post-hoc* test was used to analyse Ca^{2+} transients and sarcomere shortening amplitudes. A Kruskal-Wallis followed by a Dunn's *post hoc* test was used to compare relaxation kinetics.

Figure 3. Simultaneous PDE4 and PDE3 inhibition promotes pro-arrhythmogenic spontaneous calcium waves in APVMs

(A) Representative traces of Ca^{2+} transients in Fura-2 loaded APVMs paced at 1 Hz. The occurrence of spontaneous Ca^{2+} waves (SCWs) was evaluated during a 10 s pause in pacing. This protocol was repeated in basal conditions (Ctrl), during PDE4 or PDE3 inhibition by either Ro 20-1724 (Ro) or cilostamide (Cil) respectively, and upon concomitant PDE4 and PDE3 inhibition (Ro + Cil). **(B)** Average number of SCWs (± SEM) in all conditions tested. Numbers indicate the number of cells obtained from 4 pigs. **(C)** Proportion of arrhythmic cells for all conditions tested. Numbers indicate the number of arrhythmic cells over all cells assessed in each condition. Statistical significance is indicated as: *, $p<0.05$; **, $p<0.01$. A Kruskal-Wallis followed by a Dunn's *post hoc* test was used to compare the occurrence of SCWs in the different

experimental conditions and a Chi^2 test followed by a Fischer exact test was used to compare the percent of arrhythmic cells.

Figure 4. Concomitant PDE3 and PDE4 inhibition leads to inotropic, lusitropic and the occurrence of SCWs in APVMs isolated from a model of combined RV overload reproducing repaired tetralogy of Fallot

(A) Representative traces of Ca^{2+} transients and sarcomere length variation ($\Delta L/L_0$) recorded in a Fura 2- loaded APVM isolated from a repaired tetratology of Fallot (rTOF) pig and paced at 1 Hz. Basal conditions (Ctrl) and after addition of PDE4 inhibitor alone (Ro 20-1724, Ro, 10 µM) or additional PDE3 inhibition (Cilostamide, Cil, 1 µM) (Ro + Cil). **(B)** Mean amplitude (±SEM) of Ca^{2+} transients and decay kinetic (Tau ± SEM) of Ca^{2+} transients for each condition tested. **(C)** Sarcomere shortening and average relaxation kinetic (Tau ± SEM) for all conditions tested. **(D)** Representative traces of Ca^{2+} transients in Ctrl, upon Ro and Ro + Cil when exhibiting a SCW. **(E)** Average number of SCWs (± SEM) in all conditions tested. **(F)** Proportion of arrhythmic cells for all conditions tested. Numbers indicate the number of cells from 3 pigs. Statistical significance is indicated as: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; a Kruskal-Wallis followed by a Dunn's *post hoc* test was used to analyse Ca^{2+} transients, sarcomere shortening amplitudes and to compare the occurrence of SCWs in the different experimental conditions. A Chi^2 test followed by a Fischer exact test was used to compare the percent of arrhythmic cells.

Figure 5. Similarly to PDE3 inhibition, PDE4 inhibition enhances EC coupling in APVMs upon β-AR stimulation

(A,B) Representative traces of Ca^{2+} transients and sarcomere length variation ($\Delta L/L_0$) recorded simultaneously in Fura-2-loaded APVMs paced at 1 Hz in basal conditions (Ctrl), upon β-AR stimulation by Iso (10 nM) alone or with PDE4 inhibitor (Ro 20-1724, Ro, 10 µM, A) or PDE3 inhibitor (Cilostamide, Cil, 1 µM, B). **(C)** Mean amplitudes (±SEM) and relaxation kinetics Tau (± SEM) of Ca^{2+} transients and sarcomere shortening for Ctrl, Iso, and Iso + Ro. **(D)** Mean amplitude (±SEM) and relaxation kinetics Tau (± SEM) of Ca^{2+} transients and sarcomere shortening for Ctrl, Iso, and Iso + Cil. Numbers indicate the number of cells obtained from 4 pigs. Statistical significance is indicated as: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; a nested ANOVA followed by Tukey's *post-hoc* test was used to analyse Ca^{2+} transients and sarcomere shortening amplitudes. A Kruskal-Wallis followed by a Dunn's *post hoc* test was used to compare relaxation kinetics.

Figure 6. PDE4 inhibition like PDE3 inhibition, promotes pro-arrhythmogenic spontaneous calcium waves in APVMs upon β-AR stimulation

(A) Representative traces of Ca^{2+} transients in Fura-2-loaded APVMs paced at 1 Hz. The occurrence of spontaneous Ca^{2+} waves (SCWs) was evaluated during a 10 s pause in pacing. This protocol was repeated in basal conditions (Ctrl), upon β-AR stimulation with isoproterenol (Iso, 10 nM) alone or with PDE4 inhibition by Ro 20-1724 (Iso + Ro), or PDE3 inhibition by cilostamide (Iso + Cil). **(B)** Average number of SCWs (± SEM) in all conditions tested. Numbers indicate the number of cells obtained from 4 pigs. **(C)** Proportion of arrhythmic cells for all conditions tested. Numbers indicate the number of arrhythmic cells over all cells assessed for each condition. Statistical significance is indicated as: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. a Kruskal-Wallis followed by a Dunn's *post hoc* test was used to compare the occurrence of SCWs, and a Chi^2 test followed by a Fischer exact test was used to compare the percent of arrhythmic cells.

Figure 7. PDE3A and PDE4 subtypes expression in right ventricle tissue and isolated cardiomyocytes from pigs

Western blots showing PDE3A, PDE4A, PDE4B and PDE4D protein expression in right ventricle tissues **(A)** or isolated cardiomyocytes from right ventricles **(B)** of 3 different pigs. Calsequestrin (CSQ) or GAPDH were used as loading controls. For specific PDE4A and PDE4B detection, rabbit polyclonal antibodies generated against their respective C-termini were used (anti-PDE4A: AC55; anti-PDE4B: 113-4). Mouse monoclonal antibody (ICOS PDE4D) was used to specifically detect PDE4D. 15 µg proteins from protein extracts were loaded.

SUPPLEMENTAL MATERIAL

METHODS

Experimental model of combined right ventricular overload

Surgical procedure mimicking repaired Tetralogy of Fallot (rTOF) was performed on–50 to 67 days old male piglets as previously described.[1,2] After premedication with ketamine hydrochloride (15 mg/kg intramuscularly), general anaesthesia was induced with 1% propofol and cisatracurium (0,3 mg/kg each 2 hours), allowing endotracheal intubation, and maintained with isoflurane in 100% oxygen (Servo 900, Siemens-Elema AB, Solna, Sweden). Through a left thoracotomy approach, a side-biting vascular clamp was longitudinally placed across the pulmonary valve annulus without obstruction of the RV outflow tract. A pulmonary valve leaflet was excised, and the pulmonary infundibulum, annulus, and trunk were enlarged by a 2 cm–long elliptically shaped polytetrafluorethylene patch to ensure loss of valve integrity. This chronic pulmonary valve regurgitation led to a right ventricular (RV) volume overload. The RV pressure overload was achieved by pulmonary artery banding, made of umbilical tape, placed around the artery truncus and secured for a final diameter of approximately 1 cm to ensure a progressive pulmonary stenosis with animal growth. Control animals did not have sham surgery. Animals were sacrificed ~4 months (Day 164-222) after surgical procedure.

Echocardiographic analysis

Echocardiography was performed on closed-chest animals under general anesthesia in dorsal decubitus. We used commercially available Vivid E9 ultrasound machine (General Electric Medical System, Milwaukee, WI, USA) equipped with a 2.5 MHz transducer. The values of all echocardiographic parameters were obtained as the average value of three consecutive cardiac cycles during transient apnoea and were analyzed on a comprehensive workstation (EchoPAC 110.1.2, GE-Healthcare, Horten, Norway). The echocardiographic analysis used for RV morphological and functional assessment in this model was previously detailed by our group.[2] Briefly, RV morphology was assessed by RV anterior wall thickness, the right on left ventricle end-diastolic diameter ratio, the tricuspid on mitral annular diameter ratio, and RV end-diastolic (RVED) and end-systolic (RVES) areas. RV systolic function was assessed by the RV fractional area change (FAC), the Tricuspid Annular Plane Systolic Excursion (TAPSE), and the peak systolic velocity (S'). Pulmonary annulus diameter and transpulmonary gradient through the pulmonary band were both measured to evaluate the degree of pulmonary stenosis. The severity of pulmonary regurgitation was assessed using color Doppler flow.[3]

Statistical analyses

Analyses were performed with statistical software GraphPad (Prism5, GraphPadSoftware, http://www.graphpad.com). Echocardiography data were tested for normality and expressed as median and range after the result of the Shapiro-Wilk test. The 2 groups (control and operated) were compared by Mann-Whitney *U* test. Differences between the mean values of two groups of Ionoptix results were analyzed by an unpaired Student's *t*-test.

References:

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Table 1: **Echocardiographic characteristics: standard parameters of right ventricular morphology and systolic function in the porcine model of combined right ventricular overload reproducing repaired tetralogy of Fallot (rTOF)**

	Healthy	**rTOF**
Clinical characteristics		
Age (d)	161 (149 to 170)	196 (164 to 222)
Weight (kg)	45 (40 to 55)	50 (37.8 to 68)
RV dimensions		
RV_{ED} area (cm^2)	8.7 (8.0 to 11.4)	21.6 (16.7 to 26.2) *
RV_{ES} area (cm^2)	4.9 (4.4 to 6.0)	14.4 (9.3 to 15.0) *
RV/LV_{ED} diameter ratio	0.43 (0.4 to 0.53)	0.94 (0.84 to 1.51) *
T/M diameter ratio	0.74 (0.70 to 0.81)	1.23 (1.12 to 1.84) *
PA annulus diameter (cm)	1.9 (1.8 to 2.0)	2.3 (2.1 to 3.0) *
RV wall thickness (mm)	3.0 (3.0 to 3.4)	6.2 (4.6 to 7.5) *
Trans-pulmonary gradient (mmHg)		
Maximal	2.8 (2.0 to 4.0)	33.4 (25.0 to 39.7) *
Mean	1.3 (1.0 to 2.0)	19.5 (13.2 to 22.4) *
RV systolic function indices		
FAC (%)	45.3 (43.5 to 57.0)	37.0 (33.0 to 53.2)
TAPSE (mm)	20.0 (17.0 to 21.0)	14 (11.35 to 17.5) *
Peak S' (cm/s)	8.0 (8.0 to 10.0)	7.0 (5.5 to 9.1)

Data are presented as the median and interquartile range. *, $p<0.05$ rTOF group (N=7) versus controls (N=12).

ED: end-diastolic; ES: end-systolic; FAC: fractional area change; LV: left ventricle; PA: pulmonary artery; RV: right ventricle; TAPSE: tricuspid annular plane systolic excursion; T/M: Tricuspid/Mitral.

Table 2: Characteristics of sarcomere shortening and calcium transients measured simultaneously in right ventricular myocytes isolated from Control and rTOF animals

	Sarcomere Shortening (% of resting length)	**Tau relaxation (s)**	**Ca^{2+} transient amplitude (% of diastolic ratio)**	**Tau decrease of Ca^{2+} transient (s)**
Healthy	1.6 ± 0.3 (n=34)	0.2 ± 0.03 (n=33)	20.5 ± 1.8 (n=34)	0.4 ± 0.02 (n=34)
rTOF	1.3 ± 0.4 (n=19)	0.3 ± 0.04 * (n=19)	19.8 ± 1.9 (n=19)	0.51 ± 0.05 * (n=19)

Data are presented as the mean ± SEM. *, p<0.05, rTOF group (N=3) *versus* controls (N=7).

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Effect of PDE4 or PDE3 inhibition on cAMP homeostasis in APVMs isolated from the rTOF pig model

(A, B) Time courses of the CFP/YFP ratio upon PDE3 inhibition (Cilostamide, Cil, 1 µM) or PDE4 inhibition (Ro 20-1724, Ro, 10 µM) or concomitant inhibition (Ro + Cil) (A) and upon β-AR stimulation (B) by Iso (10 nM) alone or with Ro in APVMs expressing the cAMP sensor Epac-S^{H187}. **(C, D)** Mean variation (± SEM) of the normalized CFP/YFP ratio upon application of the PDE inhibitors alone (Cil, Ro) or in combination (Ro + Cil) and upon β-AR stimulation by Iso (10 nM) alone and after addition of Ro (10 µM) or Cil (1 µM). Numbers indicate the number of cells from 2 pigs. Statistical significance is indicated as *, p<0.05; Kruskal-Wallis followed by Dunn's multiple comparison *post hoc* test.