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Differential regulation of cardiac excitation-contraction coupling by cAMP phosphodiesterase subtypes

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Short title: cAMP PDEs and cardiac EC coupling

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Abstract

Aims  Multiple phosphodiesterases (PDEs) hydrolyze cAMP in cardiomyocytes, but the functional significance of this diversity is not well understood. Our goal here was to characterize the involvement of three different PDEs (PDE2-4) in cardiac excitation-contraction coupling (ECC).

Methods and results  Sarcomere shortening and \( \text{Ca}^{2+} \) transients were recorded simultaneously in adult rat ventricular myocytes and ECC protein phosphorylation by PKA was determined by western blot analysis. Under basal conditions, selective inhibition of PDE2 or PDE3 induced a small but significant increase in \( \text{Ca}^{2+} \) transients, sarcomere shortening, and troponin I phosphorylation whereas PDE4 inhibition had no effect. PDE3 inhibition, but not PDE2 or PDE4, increased phospholamban phosphorylation. Inhibition of either PDE2, 3 or 4 increased phosphorylation of the myosin binding protein C, but neither had an effect on L-type \( \text{Ca}^{2+} \) channel or ryanodine receptor phosphorylation. Dual inhibition of PDE2 and PDE3 or PDE2 and PDE4 further increased ECC compared to individual PDE inhibition but the most potent combination was obtained when inhibiting simultaneously PDE3 and PDE4. This combination also induced a synergistic induction of ECC protein phosphorylation. Submaximal \( \beta \)-adrenergic receptor stimulation increased ECC and this effect was potentiated by individual PDE inhibition with the rank order of potency PDE4=PDE3>PDE2. Identical results were obtained on ECC protein phosphorylation.

Conclusions  Our results demonstrate that PDE2, PDE3 and PDE4 differentially regulate ECC in adult cardiomyocytes. PDE2 and PDE3 play a more prominent role than PDE4 in regulating basal cardiac contraction and \( \text{Ca}^{2+} \) transients. However, PDE4 becomes determinant when cAMP levels are elevated, for instance upon \( \beta \)-adrenergic stimulation or PDE3 inhibition.

Keywords  Phosphodiesterase • Excitation-contraction coupling • cAMP • Protein phosphorylation
1. Introduction

The cAMP pathway is critical for autonomic regulation of the heart. Sympathetic stimulation increases myocardial contractility mainly through stimulation of \( \beta \)-adrenergic receptors (\( \beta \)-ARs), elevation of intracellular cAMP ([cAMP]\_i) and activation of the cAMP-dependent protein kinase (PKA). PKA in turn phosphorylates key components of cardiac excitation-contraction coupling (ECC) such as the L-type Ca\(^{2+} \) channel (Ca\(_{1.2} \)), ryanodine receptor type 2 (RyR2), phospholamban (PLB), troponin I (TnI) and myosin-binding protein C (MyBP-C). The phosphorylation of Ca\(_{1.2} \) and RyR2 leads to enhanced Ca\(^{2+} \) influx and sarcoplasmic reticulum (SR) Ca\(^{2+} \) release, contributing to enhanced Ca\(^{2+} \) transients. PLB phosphorylation increases SR Ca\(^{2+} \) uptake by the Ca\(^{2+} \)-ATPase (SERCA2), thus accelerating cardiac relaxation but also increasing SR Ca\(^{2+} \) load and consequently SR Ca\(^{2+} \) release. The phosphorylation of TnI and MyBP-C reduces myofilament Ca\(^{2+} \) affinity and increases crossbridge kinetics. Altogether, these events produce the typical inotropic and lusitropic effects of \( \beta \)-AR stimulation.\(^1 \)

Intracellular cAMP levels result from the balance between cAMP synthesis by adenylyl cyclases and cAMP degradation by cyclic nucleotide phosphodiesterases (PDEs). PDEs are subdivided into 11 families among which four can 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; and PDE4.\(^2 \) While PDE1 and PDE2 can hydrolyse both cAMP and cGMP, PDE3 preferentially hydrolyses cAMP and PDE4 is specific for cAMP. Another PDE, named PDE8A which specifically hydrolyses cAMP, was shown recently to control ECC in mouse cardiac myocytes.\(^3 \) All PDE isoforms except PDE8A\(^4,5 \) are inhibited by xanthine derivatives such as 3-isobutyl-1-methylxanthine (IBMX), and a number of drugs have been developed as selective PDE inhibitors\(^6,7 \): EHNA\(^8 \) and Bay 60-7550\(^9 \) for PDE2; milrinone, cilostamide and other
bipyridines for PDE3; rolipram and Ro 20-1724 for PDE4. There is currently no commercially available selective inhibitor of PDE1.

Direct monitoring of cAMP in living cardiac myocytes emphasized the importance of PDE isoforms 2 to 4 for the control of \([cAMP]_i\). In rodent heart, a predominance of PDE4 over other PDEs for the control of cAMP signals generated by stimulation of \(\beta\)-ARs and other \(G_\text{s}\)PCRs was observed. PDE4 was shown to associate with \(\beta\)-ARs and ECC proteins, either directly or through scaffold proteins such as \(\beta\)-arrestins or A-kinase anchoring proteins. In mouse heart, a specific PDE4 variant (PDE4D3) was shown to control the phosphorylation of RyR2 and regulates diastolic SR Ca\(^{2+}\) leak. Similarly, a PDE4D variant was found to coimmunoprecipitate with SERCA2 in mouse and to control PLB phosphorylation and Ca\(^{2+}\) transients decay kinetics.

As Ca\(_{\text{v}}\)1.2 is a well characterized target of the cAMP/PKA pathway in heart, the cardiac L-type Ca\(^{2+}\) channel current (I\(_{\text{Ca,L}}\)) has been frequently used as a functional index of the contribution of PDE isoforms to this pathway. Inhibition of PDE2, PDE3 and PDE4 was shown to increase I\(_{\text{Ca,L}}\) amplitude in a number of species. Recently, we observed in mouse heart that a PDE4B variant associates with Ca\(_{\text{v}}\)1.2 and controls its activity upon \(\beta\)-AR stimulation. However, important differences exist among species as to whether PDE3 or PDE4 is predominant and whether all three PDE isoforms control the I\(_{\text{Ca,L}}\) amplitude at basal or only upon \(\beta\)-AR stimulation. PDE3 inhibitors were also reported to increase SR Ca\(^{2+}\) uptake and SR Ca\(^{2+}\) content, an effect attributed to an increase in PKA-dependent phosphorylation of PLB.

While it is well established that PDE3 inhibition exerts a direct positive inotropic effect in the heart from large mammals, the contribution of other PDE families to the regulation of cardiac contractility remains less clear and often controversial. For instance, while selective inhibition of PDE2 or PDE4 was shown to increase cardiac contractility following \(\beta\)-AR stimulation in some studies, other studies failed to demonstrate an effect.


In an attempt to reconcile these contradictory findings, we compared, in the same mammalian cardiac preparation (adult rat ventricular myocytes), the effect of a selective inhibition of PDE2, PDE3 and PDE4 on Ca\(^{2+}\) transients, sarcomere shortening, and phosphorylation of five key ECC proteins (TnI, Ca\(_{\alpha}1.2\), RyR2, PLB and MyBP-C). Furthermore, we compared for each PDE isoform, the effects observed under basal condition and after sub-maximal β-AR stimulation with isoprenaline. Our study sheds new light on the respective contribution of the three major cardiac PDE isoforms in the regulation of cardiac ECC.

2. Methods

All experiments were carried out according to the European Community guiding principles in the care and use of animals (86/609/CEE, CE Off J n°L358, 18 December 1986), the local ethics committee (CREEA Ile-de-France Sud) guidelines and the French decree n°87-848 of October 19, 1987 (J Off Rép Fr, 20 October 1987, pp. 12,245–12,248). Authorizations to perform animal experiments according to this decree were obtained from the Ministère Français de l’Agriculture, de la Pêche et de l’Alimentation (n° 92-283, June 27, 141 2007).

2.1 Reagents

Bay 60-7550 (Bay, 2-[(3,4-dimethoxyphenyl)methyl]-7-[(1R)-1-hydroxyethyl]-4-phenylbutyl]-5-methyl-imidazo[5,1-f][1,2,4]triazin-4(1H)-one) was from Alexis Biochemicals (Lausen, Switzerland): it blocks PDE2 with an IC\(_{50}\) value of 4.7 nM\(^{40}\) and was used here at a 100 nM concentration to fully block the enzyme.\(^9\) Cilostamide (Cil) was from Tocris Bioscience (Bristol, UK): it blocks PDE3 with an IC\(_{50}\) ranging from 5 nM\(^{41}\) to 27 nM\(^{42}\) 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 IC\(_{50}\) value around 1
and was used here at 10 µM. At these concentrations, Bay, Cil and Ro were shown to be selective for PDE2, PDE3 and PDE4 respectively (Supplemental Table 1). IBMX (3-isobutyl-1-methylxanthine) and isoprenaline (Iso) were from Sigma Aldrich (Saint Quentin, France).

2.2 Cardiomyocyte isolation and culture

Male Wistar rats (250–300 g) were subjected to anesthesia by intraperitoneal injection of pentothal (0.1 mg/g) and hearts were excised rapidly. Individual adult rat ventricular myocytes (ARVMs) were obtained by retrograde perfusion of the heart as previously described. For enzymatic dissociation, the hearts were perfused retrogradely at a constant flow of 6 mL/min at 37°C with a Ca²⁺-free Ringer solution containing (in mM) NaCl 117, KCl 5, NaHCO₃ 4.4, KH₂PO₄ 1.5, MgCl₂ 1.7, D-glucose 11.7, Na-phosphocreatine 10, taurine 20 and HEPES 21, pH 7.1 during 5 min, followed by a perfusion at 4 mL/min for 1 h with the same solution containing 1 mg/mL collagenase A (Boehringer, Mannheim, Germany) and 300 µM EGTA and CaCl₂ to adjust free Ca²⁺ concentration to 20 µM. The ventricles were then separated from atria, finely chopped and gently agitated to dissociate individual cells. The resulting cell suspension was filtered on a gauze and the cells were allowed to settle down. The supernatant was discarded and cells resuspended four more times with Ringer solution at increasing [Ca²⁺] from 20 µM to 300 µM. Freshly isolated cells were suspended in minimal essential medium (MEM: M 4780; Sigma, St Louis, MO USA) containing 1.2 mM [Ca²⁺] supplemented with 2.5% fetal bovine serum (FBS, Invitrogen, Cergy-Pontoise, France), 1% penicillin-streptomycin, 20 mM HEPES (pH 7.6) and plated on 35 mm, laminin-coated culture dishes (10 µg/mL) at a density of 10⁴ cells per dish. After 1 h the medium was replaced by 300 µL of FBS-free MEM. All experiments were performed on primary cell cultures 24h after plating.
2.3 Measurements of Ca$^{2+}$ transients and sarcomere shortening

Isolated cardiomyocytes were loaded with 5 µM Fura-2 AM (Invitrogen) at room temperature for 15 min and then washed with Ringer solution containing (in mM) NaCl 121.6, KCl 5.4, NaHCO$_3$ 4.013, NaH$_2$PO$_4$ 0.8, HEPES 10, glucose 5, Na pyruvate 5, MgCl$_2$ 1.8, CaCl$_2$ 1.8, pH 7.4. The loaded cells were field stimulated (5 V, 4 ms) at a frequency of 0.5 Hz. Sarcomere length and Fura-2 ratio (measured at 512 nm upon excitation at 340 nm and 380 nm) were simultaneously recorded using an IonOptix System (IonOptix, Milton, MA, USA). Briefly, myocytes were imaged at 240 Hz using an IonOptix Myocam-S CCD camera. Images were displayed within the IonWizard acquisition software (IonOptix). The myocyte of interest was aligned horizontally, parallel to the field of view. The sarcomere length was measured from a user-defined region of interest that included at least 7 sarcomeres to maximize the accuracy of the measurements. The IonWizard software determined a density trace corresponding to the alternating dark and light A- and I-bands of the cardiomyocyte, and analyzed the periodicity in the density trace by calculation of a fast Fourier transform. This analysis allowed a direct real-time measurement of sarcomere length.

The measurements of Ca$^{2+}$ transients and sarcomere shortening were performed on individual myocytes superfused for few minutes with control Ringer solution and then challenged with PDE inhibitors (single: Bay, 100 nM; Cil, 1 µM; Ro, 10 µM or IBMX, 100 µM; or combinations: Bay + Ro, Bay + Cil or Cil + Ro) during 15 minutes or with 1 nM Iso during 10 min followed by 10 min treatment with PDE inhibitors (Bay, Cil or Ro). Sarcoplasmic reticulum (SR) Ca$^{2+}$ load was measured by rapid application of caffeine (10 mM) to ARVMs after pacing at 0.5 Hz in control Ringer solution or after Cil or Cil+Ro application. Fractional Ca$^{2+}$ release was calculated as the ratio between the Ca$^{2+}$ transient amplitude and the caffeine transient amplitude (SR Ca$^{2+}$ load) measured in the same ARVM.
2.4 Cell extracts and western blot analysis

Prior to cell homogenization and biochemical analysis, intact ARVMs were treated at room temperature for 15 min with PDE inhibitors (single: Bay, 100 nM; Cil, 1 µM; Ro, 10 µM or IBMX, 100 µM; or combinations: Bay + Ro, Bay + Cil or Cil + Ro), or for 10 min with 1 nM Iso followed by 10 min treatment with PDE inhibitors (Bay, Cil or Ro). To prepare whole ARVM cell lysates, cells were lysed in cold HNTG lysis buffer containing (in mM): Hepes 50 (pH 7.5), NaCl 400, NaF 100, Na-pyrophosphate 10, MgCl₂ 1.5, EGTA 1, 10% Glycerol, 1% Triton X100 supplemented with 1 mM Na₃VO₄, and a protease inhibitor mixture (1 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 1 mM benzamidine, 10 µg/mL soybean trypsin inhibitor, 10 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL antipain, 1 µg/mL pepstatin). The lysates were centrifuged at 10,000 g for 10 min and assayed for protein concentration using bicinchoninic acid-protein reagent (Sigma Aldrich, Saint Quentin, France).

For immunoblotting assays, proteins were resolved by 4–12% or 8–16% SDS-PAGE (Lonza Verviers, Verviers, Belgium), transferred onto nitrocellulose membranes, and stained with 3% Ponceau S (Sigma Aldrich, Saint Quentin, France). The membranes were saturated with 3% bovine serum albumin. Phosphorylation of phospholamban (PLB) at Ser¹⁶ and cardiac troponin I (TnI) at Ser²³/²⁴ were detected using specific antibodies from Cell Signaling Technology (Beverly, MA, USA). Phosphorylation of ryanodine receptor (RyR2) at Ser²⁸⁰⁹ was detected using a specific antibody kindly provided by Dr A. R. Marks (Columbia University, USA). L-type Ca²⁺ channel pore forming subunit (Caᵥ1.2) phosphorylation at Ser¹⁹²⁸ was detected using the anti-CH3-P antibody.⁴⁵ Phosphorylation of myosin binding protein C (MyBP-C) at Ser²⁸² was detected using antibodies from Alexis Biochemicals (San Diego, CA, USA). Immunoreactive proteins were revealed using a 1:30,000 dilution of anti-rabbit or anti-mouse IgG peroxidase conjugate antibodies (Promega Corp., Madison, USA), and were visualized with Supersignal West Pico reagent (Perbio Science, Vigneux, France). The membranes were
stripped and reprobed with specific antibodies against calsequestrin (CSQ) (Affinity BioReagents, Ozyme, Saint-Quentin-Fallavier, France) used as a loading control. Each sample was normalized to CSQ (phosphorylated proteins/CSQ ratio) and double-normalized to the control ratio. Densitometric analyses of western blots were performed using the NIH ImageJ software.

2.5 Data Analysis

Cell contraction was assessed by the percentage of sarcomere shortening, which is the ratio of twitch amplitude (difference of end-diastolic and peak systolic sarcomere lengths) to end-diastolic sarcomere length (SL). Ca$^{2+}$ transient amplitude was assessed by the percentage of variation of the Fura-2 ratio, by dividing the twitch amplitude (difference of end-diastolic and peak systolic ratios) to end-diastolic ratio. Relaxation was assessed by measuring the time-to-50% relaxation from the time to peak shortening, and the Ca$^{2+}$ transient decay was evaluated by measuring the time-to-50% decay of the Fura-2 ratio from the time to peak ratio. These parameters were obtained by analyzing the 10 last contractions and calcium transients before addition of the next drug. All parameters were calculated offline on a dedicated software (IonWizard 6x, IonOptix). All results are expressed as mean±SEM. GraphPad Prism software (GraphPad software Inc., La Jolla, CA, USA) was used for statistical analysis. To determine statistical significance with multiple groups, we used a One-way ANOVA followed by a Newman-Keuls test for multiple comparisons. Differences with $p$ values <0.05 were considered as statistically significant. The number of independent experiments performed is indicated in the figure legends.

3. Results

3.1 Regulation of ECC by PDE2, PDE3 and PDE4 in ARVMs
To investigate the functional consequences of individual PDE inhibition on basal and β-AR stimulated ECC in ARVMs, Ca^{2+} transients and sarcomere shortening were simultaneously recorded in Fura-2-loaded ARVMs. In quiescent myocytes, the average diastolic sarcomere length (SL) was 1.68±0.01 µm (n=95) (Supplemental Table 2). Upon pacing at 0.5 Hz, the average peak amplitude of Ca^{2+} transients was 22±0.1% above the diastolic Fura-2 ratio (Supplemental Table 3), whereas sarcomere length decreased by 0.78±0.05% (n=95) (Supplemental Table 1). The relaxation kinetics of both parameters, as estimated by the time to 50% decay (t_{1/2 off}), were on average 0.43±0.01 s for Ca^{2+} transients and 0.49±0.02 s for cell contraction (n=95). On average, application of 1 nM Iso increased the amplitude of Ca^{2+} transients by 257±20.3% and sarcomere shortening by 1033.8±61.2%, (n=62, p<0.001 vs. control). Iso also significantly accelerated the relaxation phases of both signals, decreasing t_{1/2 off} for Ca^{2+} decay by 38.4±1.6% and t_{1/2 off} for contraction by 56.3±1.3% (n=62, p<0.001 vs. control). As shown in Supplemental Figure 1, the Iso response on Ca^{2+} transients and cell shortening was stable during the 10 min incubation period suggesting that β-AR stimulation by 1 nM Iso does not induce desensitization of the receptors, in our experimental conditions.

In a first series of experiments, the effects of PDE2 inhibition were tested (Fig. 1 and Supplemental Table 2 and 3) using the selective inhibitor Bay. As shown on the representative traces in Fig. 1A and on the summary data in Fig. 1B, 100 nM Bay increased basal Ca^{2+} transient amplitude and basal cell contraction approximately 2-fold. Bay also accelerated the decay kinetics of both signals, as shown by a significant decrease in the t_{1/2 off} values for both parameters (Fig. 1C). These effects were small when compared to that of 1 nM Iso. To examine whether PDE2 modulates ECC under β-AR stimulation, 100 nM Bay was applied on top of Iso. As shown in Fig. 1A and on the Supplemental Table 2 and 3, the Iso effect was potentiated upon PDE2 inhibition. However, the decay kinetics of the signals were unchanged compared to those obtained under Iso stimulation (Fig. 1C).
We next studied the consequences of a selective PDE3 inhibition on Ca\(^{2+}\) transients and cell contraction using Cil (Fig. 2 and Supplemental Table 2 and 3). Application of 1 µM Cil alone induced a significant increase in Ca\(^{2+}\) transient and sarcomere shortening amplitudes by approximately 2- and 3-fold, respectively (Fig. 2A and 2B). Cil also significantly accelerated the decay kinetics of both signals (Fig. 2C). This was accompanied by an increase in SR Ca\(^{2+}\) load with no change in SR fractional Ca\(^{2+}\) release (Fig. 5). PDE3 inhibition also strongly potentiated the effect of Iso on both parameters, enhancing their amplitude by ~30% for Ca\(^{2+}\) transient and 70% for cell shortening (Fig. 2B), and accelerating their relaxation kinetics (Fig. 2C).

In contrast to the results obtained with PDE2 or PDE3 inhibitors, PDE4 inhibition by 10 µM Ro had no effect on Ca\(^{2+}\) transients and sarcomere shortening under basal conditions (Fig. 3A and 3B and Supplemental Table 2 and 3). However, Ro strongly potentiated the effects of Iso (1 nM), further increasing the Ca\(^{2+}\) transients amplitude and sarcomere shortening by ~60% (Fig. 3B). PDE4 inhibition also significantly accelerated the decay kinetics of both signals (Fig. 3C).

We next examined the functional consequences of combinations of selective PDE inhibitors on cell contraction under basal conditions (Fig. 4 and Supplemental Table 2). As shown in Fig. 4A and B, simultaneous inhibition of PDE2 and PDE4 by a combination of 100 nM Bay and 10 µM Ro (Bay + Ro) increased Ca\(^{2+}\) transients and sarcomere shortening, and slightly accelerated the relaxation kinetics of the twitch. However, comparison with Fig. 1 shows that these effects were similar to that of individual PDE2 inhibition under basal conditions. In contrast, concomitant inhibition of PDE2 and PDE3 by 100 nM Bay and 1 µM Cil strongly increased the amplitude of Ca\(^{2+}\) transients and sarcomere shortening and these effects were significantly larger than when either PDE2 or PDE3 alone were inhibited (p<0.05 versus Bay and Cil alone). The relaxation kinetics were significantly accelerated as compared
to control (Fig. 4C), t\(_{1/2}\) off values of sarcomere shortening were significantly smaller for Bay + Cil as compared to Bay alone (compare Fig. 1C and 4C) but not as compared to Cil alone (compare Fig. 2C and 4C). This suggests that PDE3 has a more prominent role in controlling cell relaxation than PDE2 under basal conditions. Concomitant inhibition of PDE3 and PDE4 by Cil + Ro had a major impact on ECC, increasing the amplitude of Ca\(^{2+}\) transients 2.5-fold and sarcomere shortening 15-fold (Fig. 4B). This was accompanied by a major acceleration of Ca\(^{2+}\) transients and cell shortening decay phases, which was significantly larger than with Bay + Cil (Fig. 4C). Furthermore, Cil + Ro doubled SR Ca\(^{2+}\) load and drastically increased fractional Ca\(^{2+}\) release from 40% in Ctrl or after PDE3 inhibition, to 80% when both PDE3 and PDE4 were inhibited (Fig. 5). Finally, application of the broad spectrum PDE inhibitor IBMX (100 µM) had similar functional effects as a concomitant inhibition of PDE3 and PDE4.

3.2 Regulation of the phosphorylation status of key ECC proteins by PDEs

To get some insights into the molecular mechanisms by which individual PDE families regulate ECC in ARVMs, we examined the consequences of PDE inhibition on the phosphorylation of key ECC proteins. For this, we performed Western blot analysis with phospho-specific antibodies directed against TnI, MyBP-C, PLB, RyR2 and Ca\(_{1.2}\) under basal conditions (Fig. 6) or after a non maximal β-AR stimulation by Iso (1 nM) (Fig. 7). In line with ECC measurements, Fig. 6 shows that global PDE inhibition with IBMX or concomitant inhibition of PDE3 and PDE4 by Cil + Ro led to a major increase in the phosphorylation of TnI (Fig. 6A), MyBP-C (Fig. 6B), PLB (Fig. 6C), RyR2 (Fig. 6D) and Cav1.2 (Fig. 6E). Individual inhibition of PDE2 and PDE3 as well as simultaneous inhibition of PDE2 and PDE3 or PDE2 and PDE4 slightly but significantly increased the phosphorylation of TnI, whereas inhibition of PDE4 alone did not (Fig. 6A). In contrast, MyBP-C phosphorylation was significantly increased by selective inhibition of PDE2, PDE3 or PDE4 and by their dual inhibition, especially in the case
of PDE3 and PDE4 (Fig. 6B). Selective PDE3 inhibition by Cil or simultaneous inhibition of PDE2 and PDE3 also increased significantly PLB phosphorylation whereas individual or associated PDE2 and PDE4 inhibition did not (Fig. 6C). The phosphorylation of Cav1.2 and RyR2 was only increased by simultaneous blockade of PDE3 and PDE4 or global PDE inhibition with IBMX (Fig. 6D and E).

Fig. 7 shows that after a non-maximal β-AR stimulation by Iso, we observed a significant increase in the phosphorylation of TnI, MyBP-C, PLB, RyR2 and Cav1.2. A maximal level of phosphorylation was obtained by treatment of cells by Iso followed by IBMX (Fig. 7). The effect of Iso on the phosphorylation of all of these proteins was potentiated by the selective inhibition of PDE4, in agreement with the results obtained on Ca\(^{2+}\) transients, sarcomere shortening and relaxation kinetics. Similar results were obtained upon PDE3 inhibition, except that Cil only tended to increase the level of phosphorylation of TnI. In contrast, PDE2 inhibition only increased the phosphorylation of MyBP-C and Cav1.2 in the presence of Iso.

4. Discussion

It is well established that cardiac ECC is regulated by the cAMP/PKA pathway. Initiation of the pathway takes place at the sarcolemmal membrane by cAMP synthesis through the activity of adenylyl cyclases; termination of the pathway involves the activity of cyclic nucleotide PDEs which are responsible for the hydrolysis of cAMP into 5′-AMP, and phosphatase activity which is responsible for PKA substrate dephosphorylation. At least five PDE families are reported to degrade cAMP in heart.\(^ {2,46}\) Comparison of the functional role of individual PDE families is possible by using pharmacological agents that selectively inhibit their activity. Since selective inhibitors of PDE1 and PDE8 are still lacking, we focused our attention on PDE2, PDE3 and PDE4 for which selective inhibitors exist. Using Bay to inhibit PDE2, Cil to inhibit PDE3 and
Ro to inhibit PDE4, as well as the broad spectrum PDE inhibitor (IBMX), we explored the role of these three PDEs in three different sets of experimental conditions (at basal, upon β-AR stimulation, and upon dual inhibition), and on nine different parameters: PKA phosphorylation level of TnI, MyBP-C, PLB, RyR2 and Ca,1.2; amplitude and relaxation kinetics of sarcomere shortening and Ca\(^{2+}\) transients. It is known that cAMP signals induced by β-AR stimulation also activate the Ca\(^{2+}\)/Calmodulin-dependent kinase II (CaMKII), which phosphorylates some of the main PKA substrates.\(^{47}\) Thus, it is conceivable that some of the effects of the PDE inhibitors are due to the activation of CaMKII. However, inhibition of this kinase by KN-93 (1 μM) did not alter the positive inotropic effects of IBMX (Supplemental Figure 2), suggesting that PKA is the main effector of the increased Ca\(^{2+}\) transients and sarcomere shortening observed upon PDE inhibition.

Our results indicate that only PDE2 and PDE3 appear to regulate ECC under basal conditions. However, upon β-AR stimulation, individual inhibition either of PDE2, PDE3 or PDE4 potentiates the β-AR response of Ca\(^{2+}\) transient and cell contraction to a similar extent. This suggests that PDE2 and PDE3 are active in basal conditions, whereas PDE4 becomes activated upon β-AR stimulation or PDE3 inhibition.

In ARVMs, biochemical and functional measurements indicate that PDE3 and PDE4 are the major PDEs degrading cAMP.\(^{12,14,23}\) Accordingly, experiments shown here indicate that PDE3 and PDE4 dominate. This is especially true for the control of the SR Ca\(^{2+}\) load (Fig. 5), which determines Ca\(^{2+}\) transient amplitude.\(^{48}\) Interestingly, while PDE3 inhibition alone does not increase fractional release (probably because it affects only SR Ca\(^{2+}\) load) the combination Cil + Ro have a tremendous effect on this parameter. This can be explained by the increased I_{Ca,L} amplitude\(^{14}\) and RyR2 open probability leading to an increase of the gain of ECC\(^{49}\) occurring only when both PDEs are inhibited. Hence, inhibition of these two PDEs induces a massive phosphorylation of ECC proteins, and a large increase in Ca\(^{2+}\) transients and sarcomere
shortening (Fig. 4, 6). Other dual combinations of PDE inhibition had much smaller effects on protein phosphorylation and ECC, indicating that PDE3 and PDE4 can compensate for each other, and that their function is partially redundant.

Although PDE2 represents only a few percents of total hydrolytic activity in ARVMs, we show here that it regulates basal and β-AR-stimulated ECC. This is consistent with previous studies showing that PDE2 modulates cAMP levels and β-AR responses in rodent cardiac myocytes. In particular, PDE2 modulates the β-AR regulation of the L-type Ca\(^{2+}\) current (I\(_{\text{Ca,L}}\)) in cardiac myocytes thus providing a possible mechanism for the effects of PDE2 inhibition on Iso-stimulated Ca\(^{2+}\) transients and contraction. In contrast, the mechanism by which PDE2 regulates basal ECC is less clear since PDE2 inhibition has no effect on basal I\(_{\text{Ca,L}}\) in ARVMs. MyBP-C could participate in the effects of PDE2 on basal ECC by increasing cross bridge cycling and myofilament Ca\(^{2+}\) sensitivity.

In contrast to PDE4, selective PDE3 inhibition increased the amplitude and relaxation kinetics of Ca\(^{2+}\) transients and sarcomere shortening under basal conditions (Fig. 2), and this was associated with enhanced phosphorylation of PLB, TnI and MyBP-C but not Ca\(_{\text{v}}\)1.2 or RyR2 (Fig. 6). These data are in line with previous findings showing a subcellular localization of PDE3 in SR-enriched membrane fraction and the positive effects of PDE3 inhibitors on SR Ca\(^{2+}\) uptake and SR Ca\(^{2+}\) content. This is also consistent with the lack of effect of selective PDE3 inhibition on basal I\(_{\text{Ca,L}}\) in ARVMs.

In the present study, we established that PDE2, PDE3 and PDE4 have distinct roles in controlling ECC. In the absence of hormonal stimulation, when intracellular cAMP concentration is low, PDE2 and especially PDE3 are dominant to control cardiac contraction. PDE3 contribution to the degradation of basal cAMP could be explained by its high affinity for cAMP (K\(_{\text{m}}\) < 1\(\mu\)M). For PDE2, the scheme must be different because of its low affinity for cAMP. Nonetheless, under basal conditions, localized production of cGMP may occur to
activate PDE2 by binding to its regulatory GAF-B domain.\(^{54}\) PDE2 activation mediated by cGMP could exacerbate its role in controlling cAMP levels regulating ECC as recently reported in cardiomyocytes.\(^{55}\) When cAMP levels are increased, PDE4 turns on and becomes crucial to control global cAMP homeostasis and ECC. However, despite a modest effect on cAMP level, inhibition of PDE2 and PDE3 also enhances β-AR inotropic and lusitropic effects, demonstrating that PDE2, PDE3 and PDE4 act in concert to modulate β-AR stimulation of cardiac contraction. Interestingly, although cardiotonic drugs such as milrinone produce beneficial inotropic effects upon acute administration,\(^{56}\) these agents increase mortality in patients with dilated cardiomyopathy upon chronic treatment.\(^{57}\) A challenge of future research will be to determine the molecular mechanisms that underlie long term detrimental effects of PDE inhibitors in heart failure in order to design new therapeutic approaches to enhance the beneficial effects and suppress detrimental long term effects of PDE inhibitors.

5. Limitations

The present study focused on the role of three different PDE families in controlling the ECC in isolated ARVMs. It is known that the expression level of these PDEs varies among different species and is dependent on the development stage and on the cardiac territory investigated.\(^{32}\) However, PDE expression is relatively conserved between murine models and human.\(^{58}\) In mammalian heart, PDE3 and PDE4 remain the two major enzymes degrading cAMP and controlling cardiac ECC. Thus, even if our results might not be fully transposable to any cellular cardiac models or human heart, we unveil how cardiac function is finely tuned by these PDE families. Furthermore, their expression and distribution are altered under pathophysiological conditions.\(^{59}\) Therefore, the relative contribution of PDE2, PDE3 and PDE4 to compartmentalize cAMP signals and maintain specific PKA phosphorylation of the key ECC
proteins, is most probably differently affected in various cardiac diseases. Future studies investigating their participation in pathological conditions are necessary to design new therapeutic strategies targeting PDEs.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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Figure Legends

Figure 1  Effect of PDE2 inhibition on ECC in ARVMs. (A) Representative traces of Ca^{2+} transients (top) and sarcomere shortening (bottom) recorded in ARVMs paced at 0.5 Hz and loaded with Fura-2 AM (5 µM) showing the effect of PDE2 inhibition by Bay (0.1 µM) (in black) on basal conditions (dashed lines) or upon β-adrenergic stimulation by 1 nM Iso (in gray). (B) Mean amplitude (± SEM) of Ca^{2+} transients (upper graph, in % of diastolic ratio) and sarcomere shortening (lower graph, in % of resting sarcomere length, SL) in control (white bar, n=24 cells, 13 rats), Bay (black bar, n=24 cells, 13 rats), Iso (dark gray bar, n=11 cells, 4 rats) and Iso + Bay (light gray bar, n=11 cells, 4 rats). C Average time to 50% relaxation of Ca^{2+} transients (upper graph) and sarcomere shortening (lower graph) in similar experimental conditions as in B. Statistical significance is indicated as: *, p<0.05; **, p<0.01; ***, p<0.001; and ###, p<0.001 (One-way ANOVA, Newman-Keuls).

Figure 2  Effect of PDE3 inhibition on ECC in ARVMs. (A) Typical traces of Ca^{2+} transients (top) and sarcomere shortening (bottom) in Fura-2 loaded ARVMs electrically stimulated (0.5 Hz) representing the effect of PDE3 inhibition by cilostamide (Cil, 1 µM) (in black) under basal conditions (dashed lines) or during isoprenaline stimulation (Iso, 1 nM) (in gray). (B) Mean amplitude (± SEM) of Ca^{2+} transients (upper graph) and sarcomere shortening (lower graph) in control conditions (white bar, n=16 cells, 7 rats), Cil (black bar, n=16 cells, 7 rats), Iso (dark gray bar, n=9 cells, 3 rats) and Iso + Cil (light gray bar, n=9 cells, 3 rats). (C) Average time to 50% relaxation for Ca^{2+} transients (upper graph) and sarcomere shortening (lower graph) are presented for each conditions tested. Statistical significance is indicated as: *, p<0.05; ***, p<0.001; and *, p<0.05; ###, p<0.001 (One-way ANOVA, Newman-Keuls).

Figure 3  Effect of PDE4 inhibition on ECC in ARVMs. (A) Individual traces of Ca^{2+} transients (top) and sarcomere shortening (bottom) in Fura-2 loaded ARVMs paced at 0.5 hz representing the effect of PDE4 inhibition by Ro (10 µM) (in black) under basal conditions (dashed lines) or after isoprenaline stimulation (Iso, 1 nM) (in gray). (B) Mean amplitude (± SEM) of Ca^{2+} transients (upper graph) and sarcomere shortening (lower graph) in control conditions (white bar, n=11 cells, 6 rats), Ro (black bar, n=11, 6 rats), Iso (dark gray bar, n=12 cells, 6 rats) and Iso + Ro (light gray bar, n=12 cells, 6 rats). (C) Average time to 50% relaxation for Ca^{2+} transients (upper graph) and sarcomere shortening (lower graph) are presented for
control, Ro, Iso and Iso + Ro. Statistical significance is indicated as: ***, p<0.001; and #, p<0.05; ##, p<0.01; ###, p<0.001 (One-way ANOVA, Newman-Keuls).

**Figure 4** Effect of concomitant PDE inhibition on Ca\(^{2+}\) transients and cell contraction in ARVMs under basal conditions. (A) Representative traces of Ca\(^{2+}\) transients (top) and sarcomere shortening (bottom) obtained in Fura-2 loaded ARVMs paced at 0.5 Hz showing the effect of simultaneous PDE2 and PDE4 inhibition by Bay (0.1 µM) and Ro (10 µM) (Bay + Ro); PDE2 and PDE3 inhibition by Bay and cilostamide (1 µM) (Bay + Cil); PDE3 and PDE4 inhibition by Cil and Ro (Cil + Ro); and global PDE inhibition by IBMX (100 µM). (B) Mean amplitude (± SEM) of Ca\(^{2+}\) transients (upper graph) and sarcomere shortening (lower graph) in control conditions (white bar), or after concomitant PDE inhibition by Bay + Ro (light gray bar, n=14 cells, 5 rats), Bay + Cil (dark gray bar, n=11 cells, 4 rats), Cil + Ro (black bar, n=9 cells, 4 rats) and IBMX (hatched bar, n=10 cells, 3 rats). (C) Mean (± SEM) time to 50% relaxation for Ca\(^{2+}\) decay (upper graph) and sarcomere shortening (lower graph) the same conditions as in B. Statistical significance is indicated as: *, p<0.05; **, p<0.01; ***, p<0.001; and #, p<0.05; ###, p<0.001 (One-way ANOVA, Newman-Keuls).

**Figure 5** Effect of concomitant PDE3 and PDE4 inhibition on sarcoplasmic reticulum Ca\(^{2+}\) load and fractional release in ARVMs. (A) Representative traces of Ca\(^{2+}\) transients obtained with caffeine (10 mM) recorded in Fura-2 loaded ARVMs after pacing at 0.5 Hz, under basal conditions (Ctrl), upon PDE3 inhibition by cilostamide (Cil, 1 µM) or PDE3 and PDE4 inhibition by Cil and Ro (10 µM) (Ro + Cil). (B) Mean amplitude (± SEM) of calcium transients induced by caffeine (10 mM) estimating sarcoplasmic reticulum Ca\(^{2+}\) content in Ctrl (white bar, n=8 cells, 3 rats), Cil (grey bar, n=8 cells, 3 rats), and Cil + Ro (black bar, n=9 cells, 3 rats). (C) Average fractional release (± SEM) in Ctrl (white bar, n=8 cells, 3 rats), Cil (grey bar, n=8 cells, 3 rats), and Cil + Ro (black bar, n=9 cells, 3 rats). Statistical significance is indicated as: *, p<0.05; ***, p<0.001 (versus control cells) and ###, p<0.001 (versus Cil treated cells) (One-way ANOVA, Newman-Keuls).

**Figure 6** Effect of PDE inhibitors on phosphorylation of key ECC proteins in basal conditions. Unstimulated ARVMs were treated or not with Ro (10 µM), cilostamide (Cil, 1 µM), Ro + Cil, Bay (0.1 µM), Bay + Cil, Bay + Ro and IBMX (100 µM) as indicated for 15 min. Whole proteins were extracted and 50 µg of proteins were analysed by Western blot using antibodies for phospho-troponin I (P-TnI) (A), phospho-myosine-binding protein C (P-MyBP-
C) (B), phospho-phospholamban (P-PLB) (C) phospho-ryanodine receptor (P-RyR2) (D) and phospho-Cav1.2 (E). The membranes were stripped and reprobed with calsequestrin antibodies (CSQ) used as a loading control. A representative blot is shown, and at least 5 separate experiments were performed (n=6 for P-TnI, n=5 for P-MyBP-C, n=6 for P-PLB, n=5 for P-RyR and n=5 for P-Cav1.2), giving similar results. Phosphorylated proteins/CSQ ratios were quantified, expressed as means ± SEM and normalized to untreated ARVMs (Ctrl). Statistical significance is indicated as: *, p<0.05; **, p<0.01; ***, p<0.001 (One-way ANOVA, Newman-Keuls).

Figure 7 Effect of PDE inhibition on the phosphorylation of key ECC proteins under β-AR stimulation. ARVMs were treated for 10 min with isoprenaline (1 nM, Iso) followed by 10 min treatment with PDE inhibitors Bay (0.1 µM), Cil (1 µM), Ro (10 µM) or IBMX (100 µM). Representative Western blots of cardiac myocyte lysates probed for phospho-troponin I (P-TnI) (A), phospho-myosine-binding protein C (P-MyBP-C) (B), phospho-phospholamban (P-PLB) (C) phospho-ryanodine receptor (P-RyR2) (D) and phospho-Cav1.2 (E). The membranes were stripped and reprobed with calsequestrin (CSQ) antibodies used as a loading control. A representative blot is shown, and at least 5 separate experiments were performed (n=5 for P-TnI, n=7 for P-MyBP-C, n=6 for P-PLB, n=6 for P-RyR and n=5 for P-Cav1.2), giving similar results. Phosphorylated proteins/CSQ ratios were quantified, expressed as means ± SEM and normalized to untreated ARVMs (Ctrl). Statistical significance is indicated as: *, p<0.05; **, p<0.01; ***, p<0.001; and #, p<0.05; ##, p<0.01; ###, p<0.001 (One-way ANOVA, Newman-Keuls).
Figure 1

(A) Graph showing Fura-2 ratio (% of diastolic ratio) and Sarcomere shortening (% of resting SL) for different treatments:
- Ctrl
- Iso
- Iso + Bay

(B) Bar graph showing Fura-2 ratio (% of diastolic ratio) for the following conditions:
- Ctrl
- Bay
- Iso
- Iso + Bay

(C) Bar graph showing $T_{1/2}$ off relaxaton (s) for the following conditions:
- Ctrl
- Bay
- Iso
- Iso + Bay

Significance levels indicated by stars:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001
- ### p < 0.0001
Figure 2
Figure 3
Figure 4

(A) Graph showing the Fura-2 ratio (% of diastolic ratio) and Sarcomere shortening (% of resting SL) for different treatments: Ctrl, Bay + Ro, Bay + Cil, Cil + Ro, IBMX. The traces indicate relaxation over time with 200 ms markers.

(B) Bar graph displaying the Fura-2 ratio and T_{1/2 off} relaxation (s) for each treatment group. Significant differences are indicated by asterisks (**, ***).

(C) Bar graph showing the T_{1/2 off} relaxation (s) for each treatment group with similar significance markers.

Figure 4
Figure 5

A

Ctrl

Caffeine

Caffeine

5 s

Cil

Caffeine

Cil + Ro

Fura-2 ratio
(% of diastolic ratio)

0

40

80

120

160

200

240

B

C

Fura-2 ratio
(% of diastolic ratio)

0

40

80

120

160

200

240

Ctrl

Cil

Cil + Ro

Fractional Release

0.0

0.2

0.4

0.6

0.8

1.0

Ctrl

Cil

Cil + Ro

* ***

***

Figure 5
**Figure 6**

(A) P-Tnl / CSQ (normalized to Ctrl)

(B) P-MyBP-C / CSQ (normalized to Ctrl)

(C) P-PLB / CSQ (normalized to Ctrl)

(D) P-RyR2 / CSQ (normalized to Ctrl)

(E) P-Cav1.2 / CSQ (normalized to Ctrl)
Figure 7
Supplementary Material

Differential regulation of cardiac excitation-contraction coupling by cAMP phosphodiesterase subtypes

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Supplemental Figure 1  Representative traces of Ca\textsuperscript{2+} transients and sarcomere shortening of ARVMs. Raw traces of Ca\textsuperscript{2+} transients (A) and sarcomere shortening (B) simultaneously recorded in individual Fura-2 loaded ARVMs paced at 0.5 Hz. The solid bars indicate application of isoprenaline (Iso, 1 nM) and Iso+Bay 60-7550 (100 nM).
**Supplemental Figure 2** The effect of PDE inhibition on Ca\(^{2+}\) transients and cell contraction in ARVMs is not affected by CaMKII inhibition. (A) Mean amplitude (± SEM) of Ca\(^{2+}\) transients (top, in % of diastolic ratio) and sarcomere shortening (bottom, in % of resting sarcomere length, SL) obtained in Fura-2 loaded ARVMs paced at 0.5 Hz under basal conditions (Ctrl; white bars, n=18 cells, 8 rats), upon CaMKII inhibition (KN-93, 1 µM, grey bars, n=12 cells, 3 rats), non specific PDE inhibition by IBMX (100 µM) alone (black bars, n=18 cells, 8 rats) or associated with CaMKII inhibition (KN-93 + IBMX, hatched bars, n=12 cells, 3 rats). Statistical significance is indicated as: NS, non significant; ***, p<0.001 (One-way ANOVA, Newman-Keuls).
Supplemental Table 1: Selectivity of cardiac cAMP PDE Inhibitors

| Inhibitors | PDE2  | PDE3  | PDE4  |
|------------|-------|-------|-------|
| Bay 60-7550| 0.0047 | >4    | 1.8   |
| Cilostamide| 15    | 0.042 | 80    |
| Ro 20-1724 | >200  | >200  | 2     |

The inhibition constants $K_i$ of each PDE inhibitor are reported in µM for each PDE family.
| Stimulation | Ctrl       | Bay        | Cil        | Ro         | Bay + Ro    | Bay + Cil   | Cil + Ro   | IBMX       | Iso        | Iso + Bay  | Iso + Cil | Iso + Ro  |
|-------------|------------|------------|------------|------------|-------------|-------------|------------|------------|------------|------------|-----------|-----------|
| Diastolic SL (µm) | 1.681±0.006 | 1.682±0.009 | 1.687±0.021 | 1.700±0.012 | 1.687±0.010 | 1.679±0.011 | 1.668±0.011 | 1.665±0.022 | 1.687±0.008 | 1.679±0.016 | 1.630±0.019 | 1.645±0.020 |
| Systolic SL (µm)  | 1.668±0.004 | 1.650±0.011 | 1.636±0.023 | 1.686±0.013 | 1.655±0.013 | 1.567±0.039 | 1.462±0.021 | 1.427±0.025 | 1.511±0.013 | 1.429±0.021 | 1.360±0.030 | 1.344±0.024 |
| ΔSL (µm)         | 0.013±0.001 | 0.032±0.007 | 0.051±0.013 | 0.013±0.004 | 0.032±0.008 | 0.112±0.033 | 0.206±0.019 | 0.238±0.013 | 0.175±0.012 | 0.250±0.025 | 0.270±0.021 | 0.301±0.022 |
| Sarcomere shortening (%) | 0.78±0.05  | 1.90±0.30  | 3.05±0.69  | 0.79±0.17  | 1.87±0.37  | 6.70±2.02  | 12.35±1.27 | 14.32±1.39 | 10.37±0.56  | 14.80±0.88  | 16.60±1.37  | 18.28±1.13  |
| Number of cells/rats | 95/17     | 24/4       | 16/3       | 11/6       | 14/5       | 11/4       | 9/4        | 10/3       | 62/10      | 11/4       | 9/3       | 12/6      |

Individual cardiac myocytes were superfused for few minutes with control Ringer solution and then challenged with PDE inhibitors (single: Bay 60-7550, Bay, 100 nM; cilostamide, Cil, 1 µM; Ro 20-1724, Ro, 10 µM or IBMX, 100 µM; or combinations: Bay + Ro, Bay + Cil or Cil + Ro) during 15 min or with isoprenaline (Iso, 1 nM) during 10 min followed by 10 min treatment with PDE inhibitors (Bay, Cil or Ro). Diastolic and systolic sarcomere length (SL), ΔSL (diastolic SL-systolic SL), and sarcomere shortening (ΔSL/diastolic SL*100) are expressed as mean ± SEM for each stimulation.
Individual cardiac myocytes were superfused for few minutes with control Ringer solution and then challenged with PDE inhibitors (single: Bay 60-7550, Bay, 100 nM; cilostamide, Cil, 1 µM; Ro 20-1724, Ro, 10 µM or IBMX, 100 µM; or combinations: Bay + Ro, Bay + Cil or Cil + Ro) during 15 min or with Isoproterenol (Iso, 1 nM) during 10 min followed by 10 min treatment with PDE inhibitors (Bay, Cil or Ro). Diastolic and systolic Fura-2 ratio, ΔR (systolic Fura-2 ratio-diastolic Fura-2 ratio), and Fura-2 ratio (%) (ΔR/diastolic Fura-2 ratio*100) are expressed as mean ± SEM for each stimulation.
Supplemental References

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