Calmodulin kinase II inhibition limits the pro-arrhythmic Ca\textsuperscript{2+} waves induced by cAMP-phosphodiesterase inhibitors

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Aims

A major concern of using phosphodiesterase (PDE) inhibitors in heart failure is their potential to increase mortality by inducing arrhythmias. By diminishing cyclic adenosine monophosphate (cAMP) hydrolysis, they promote protein kinase A (PKA) activity under β-adrenergic receptor (β-AR) stimulation, hence enhancing Ca\textsuperscript{2+} cycling and contraction. Yet, cAMP also activates CaMKII via PKA or the exchange protein Epac, but it remains unknown whether these pathways are involved in the pro-arrhythmic effect of PDE inhibitors.

Methods and results

Excitation–contraction coupling was investigated in isolated adult rat ventricular myocytes loaded with Fura-2 and paced at 1 Hz allowing coincident measurement of intracellular Ca\textsuperscript{2+} and sarcomere shortening. The PDE4 inhibitor Ro 20-1724 (Ro) promoted the inotropic effects of the non-selective β-AR agonist isoprenaline (Iso) and also spontaneous diastolic Ca\textsuperscript{2+} waves (SCWs). PDE4 inhibition potentiated RyR2 and PLB phosphorylation at specific PKA and CaMKII sites increasing sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} load and SR Ca\textsuperscript{2+} leak measured in a 0Na\textsuperscript{+}/0Ca\textsuperscript{2+} solution ± tetracaine. PKA inhibition suppressed all the effects of Iso ± Ro, whereas CaMKII inhibition prevented SR Ca\textsuperscript{2+} leak and diminished SCW incidence without affecting the inotropic effects of Ro. Inhibition of Epac2 but not Epac1 diminished the occurrence of SCWs. PDE3 inhibition with cilostamide induced an SR Ca\textsuperscript{2+} leak, which was also blocked by CaMKII inhibition.

Conclusion

Our results show that PDE inhibitors exert inotropic effects via PKA but lead to SCWs via both PKA and CaMKII activation partly via Epac2, suggesting the potential use of CaMKII inhibitors as adjuncts to PDE inhibition to limit their pro-arrhythmic effects.

Keywords
cAMP • 5′–3′ cyclic nucleotide phosphodiesterases • β-adrenergic receptors • Arrhythmia • CaMKII

1. Introduction

Upon stress or during exercise, norepinephrine released by sympathetic nerve terminals activates the β-adrenergic receptors (β-ARs) to elicit positive inotropic, chronotropic, and lusitropic effects. β-ARs couple primarily to G\textsubscript{s} proteins, leading to stimulation of adenylyl cyclases and cyclic adenosine monophosphate (cAMP) production. In turn, cAMP activates the cAMP-dependent protein kinase A (PKA), which phosphorylates key proteins of the cardiac excitation–contraction coupling (ECC) process, including the sarcомemal L-type Ca\textsuperscript{2+} channels (LTCCs), the ryanodine receptors (RyR2) of the sarcoplasmic reticulum (SR), and phospholamban (PLB), a constitutive inhibitor of the SR Ca\textsuperscript{2+} pump, SERCA2. As a result of PKA phosphorylation, Ca\textsuperscript{2+} entry through the sarcolemma is increased, as well as Ca\textsuperscript{2+} release and refill from the SR, leading to enhanced Ca\textsuperscript{2+} cycling and consequently to inotropic and lusitropic effects.\textsuperscript{1} A tight control of Ca\textsuperscript{2+} homeostasis is essential as perturbations such as diastolic SR Ca\textsuperscript{2+} leak via RyR2 increase occurrence of spontaneous Ca\textsuperscript{2+} waves (SCWs) leading to electrogenic sodium-Ca\textsuperscript{2+} exchanger currents, causing delayed after depolarizations which are able to trigger action potentials when the threshold for Na\textsuperscript{+} channel activation is reached.\textsuperscript{2} Along with PKA, the Ca\textsuperscript{2+}/Calmodulin-dependent kinase II (CaMKII) has been identified over the past years as a contributor to β-AR regulation of cardiac function.\textsuperscript{3} Indeed, LTCC, RyR2, and PLB are also

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substrates for CaMKII, which contributes to the inotropic and lusitropic effects of β-AR agonists. This is especially true upon excessive β-AR activation as occurring under pathological conditions, notably in heart failure (HF), in which chronic β-AR stimulation is accompanied by perturbations of the cAMP signaling pathway and increased CaMKII activity. Interestingly, CaMKII has been identified as the main suspect to provoke the Ca\(^{2+}\)-handling disturbances observed upon excessive β-AR stimulation under physiological conditions and in HF.\(^{6–11}\) Thereafter, Epac (exchange protein directly activated by cAMP), a direct target of cAMP, has emerged as a link between β-AR/cAMP signaling and CaMKII activation to promote, independently of PKA, a pro-arrhythmogenic SR Ca\(^{2+}\) leak.\(^{9–11}\)

The cAMP concentration in cells is orderly regulated not only by its synthesis but also through its degradation by cyclic nucleotide phosphodiesterases (PDEs).\(^{12}\) Among the 11 PDE families identified in mammals, PDE3 and PDE4 represent 70–90% of the total cAMP hydrolytic activity in heart.\(^{13–15}\) Given that PDE3 dominates in human heart\(^{16}\) and β-ARs are desensitized in HF, \(^{3}\) PDE3 inhibitors were identified as useful inotropes to boost the failing pump\(^{17}\) and to prevent post-operative low cardiac output syndrome after cardiac surgery.\(^{18}\) Mechanistically, PDE3 inhibitors enhance either the basal or the pre-stimulated Ca\(^{2+}\) current (depending on species and/or cardiac territory) and Ca\(^{2+}\) uptake by the SR.\(^{16,19}\) However, their use is now limited to advance disease states, mainly acute HF, as chronic treatment with these agents has been incriminated in causing arrhythmias, hence augmenting mortality of treated patients.\(^{18,20}\) PDE4 is overriding in rodent cardiomyocytes to control ECC, especially upon β-AR stimulation,\(^{13}\) but also contributes to cAMP degradation in larger species as shown in dogs\(^{21}\) and human cardiomyocytes.\(^{22}\) The pharmacological inhibition of PDE4 was shown to enhance the pro-arrhythmic effect of β-AR stimulation in mouse ventricular\(^{23}\) and human atrial strips.\(^{22}\) In mice, genetic ablation of Pde4b or Pde4d genes enhances the susceptibility to stress-induced ventricular tachycardia.\(^{24,25}\) These phenotypes were associated with exacerbated β-AR stimulation of Ca\(^{2+}\) influx in Pde4b-deficient mice\(^{24}\) and PKA-dependent hyperphosphorylation of RyR2 in Pde4d-deficient mice.\(^{25}\) In the latter, hyperphosphorylation of PLB was also reported.\(^{24,26}\)

Although the contribution of PDEs to confine PKA activity in cardiac cells is well documented, no study reported on the implication of CaMKII in the arrhythmias evoked by PDE inhibitors. Recently, a signalosome including β-AR/Epac/CaMKII organized around the scaffold protein β-arrestin was found to include a PDE4 isoform,\(^{27}\) suggesting a possible role for PDE4 in controlling this pro-arrhythmic signalling pathway. A link between PDE4 and CaMKII is also suggested by the recent finding that CaMKII can phosphorylate and activate PDE4 activity in cardiomyocytes.\(^{28}\) Collectively, these studies suggest intricate entanglement of the cAMP/PKA and cAMP/PKA/CaMKII pathways, both of which are under PDE4 control. Particularly, they suggest that Ca\(^{2+}\)-handling alterations observed upon PDE inhibition may involve CaMKII activation via the classical cAMP/PKA pathway and/or Epac. This prompted us to delineate in more detail the cellular mechanisms responsible for the pro-arrhythmic effects of PDE4 and PDE3 inhibitors and to elucidate the underlying signalling pathways of these Ca\(^{2+}\) disturbances.

### 3. Results

#### 3.1 PDE4 inhibition potentiates the inotropic effects of β-AR stimulation but promotes SCWs in ARVMs

To investigate the role of PDE4 in controlling the ECC and pro-arrhythmic effects of its inhibition, Ca\(^{2+}\)-transients (CaT) and SS were simultaneously recorded in ARVMs loaded with 1 μM Fura-2 and paced at 1 Hz (Figure 1A). Under control conditions (Ctrl), average diastolic sarcomere length was 1.75 ± 0.01 μm. Mean CaT amplitude was 38.9 ± 4.0% above the basal Fura-2 ratio, and SS was 2.1 ± 0.6%. CaT and SS declined to diastolic levels with time constants (τ) estimated at 0.33 ± 0.02 and 0.29 ± 0.03 s, respectively (Figure 1B and C). Although PDE4 inhibition with Ro 20-1724 (Ro, 10 μM) affected neither the basal amplitude of CaT nor SS, it accelerated the relaxation kinetics of both Ca\(^{2+}\) and shortening twitches to 0.26 ± 0.02 and 0.17 ± 0.02 s, respectively (P < 0.05 vs. ctrl, Figure 1C). β-AR stimulation by isoproterenol (Iso, 1 nM) increased the amplitude of CaT and SS by approximately 3- and 6-fold, respectively (P < 0.001 vs. Ctrl, Figure 1B and C). Iso also strongly accelerated the relaxation rates of both parameters by 54.5 ± 6.1 and 86.2 ± 1.4% (P < 0.001 vs. Ctrl, Figure 1C). These inotropic and lusitropic effects were potentiated by PDE4 inhibition: CaT was further increased by 19.5 ± 4.3% and SS by 39.7 ± 4.8% in Iso + Ro (P < 0.001 vs. Iso, Figure 1B). Decay time constants also tended to decrease, but this did not reach statistical significance in comparison with Iso alone (Figure 1C). When PDE4 was inhibited in the absence of β-AR agonist, the diastolic Fura-2 ratio remained unchanged and none of the cells exhibited SCWs upon cessation of stimulation (Figure 1D). When cells were subjected to Iso, only sparse SCWs were observed (0.4 ± 0.3 per 10 s) in ~20% of the cells. However, when Ro was applied in combination with Iso, a 14.7 ± 1.2% elevation of the diastolic Fura-2 ratio was observed (P < 0.001 vs. Iso), and all cells exhibited pro-arrhythmogenic SCWs at a frequency of 2.3 ± 0.2 per 10 s (P < 0.001 vs. Iso, Figure 1D and Supplementary material online, Table S1).

#### 3.2 PDE4 inhibition upon β-AR stimulation leads to diastolic SR Ca\(^{2+}\) leak

To further delineate the cellular mechanisms underlying the SCWs evoked by PDE4 inhibition upon β-AR stimulation, we measured the SR Ca\(^{2+}\) leak using a Na\(^{+}/\text{Ca}^{2+}\) exchanger to prevent Ca\(^{2+}\) extrusion by the Na\(^{+}/\text{Ca}^{2+}\) exchanger, and tetracaine (1 mM) to block RyR2s. A rapid application of 10 mM caffeine at the end of the experiment was used to evaluate SR Ca\(^{2+}\) load (Figure 2A). Although negligible

### 2. Methods

An expanded methods section is provided in the Supplementary material online.

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 (CREAA Ile-de-France Sud) guidelines, and the French decree no. 2013-118, 1 February 2013 on the protection of animals used for scientific purposes (ORF no. 0032, 7 February 2013, p2199, text no. 24). Authorizations to perform animal experiments according to this decree were obtained from the Ministère français de l’Agriculture, de l’Agroalimentaire et de la Forêt (agreement no. B 92-019-01).

Adult rat ventricular myocytes (ARVMs) were obtained using retrograde Langendorff perfusion and 1 mg/mL of collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) (37°C). An IonOptix system was used to record intracellular Ca\(^{2+}\) and sarcomere shortening (SS) simultaneously in ARVMs loaded with 1 μM Fura-2 and paced at 1 Hz, as described previously.\(^{29}\)
under basal conditions, an SR Ca\(^{2+}\) leak appeared upon β-AR stimulation with 1 nM Iso (P < 0.05 vs. Ctrl, Figure 2B), which correlated with an increased SR Ca\(^{2+}\) load (+33 ± 5.6%, P < 0.001 vs. Ctrl, Figure 2A–C). Interestingly, additional PDE4 inhibition drastically increased SR Ca\(^{2+}\) leak (+147.6 ± 33.3%, P < 0.001 vs. Iso, Figure 2A and B), whereas modestly increasing SR Ca\(^{2+}\) load (+20.6 ± 7.8%, P < 0.05 vs. Iso, Figure 2C). Fractional release (the ratio of Ca\(^{2+}\) released during a twitch divided by SR Ca\(^{2+}\) load) was increased from 52% in Ctrl to 62% in Iso (P < 0.05 vs. Ctrl) and up to 72% in Iso + Ro (P < 0.05 vs. Iso, Figure 2D).
3.3 Respective role of PKA and CaMKII in the dysregulation of Ca^{2+} homeostasis induced by PDE4 inhibition

When cells were challenged with Iso (1 nM) alone, an increase in the phosphorylation of RyR2 at Ser2808 and PLB at Thr17 (CaMKII sites) became significant only when cells were pre-incubated with KN-93 or KN-92. KN-93 also prevented the increase in diastolic Ca^{2+} levels observed in cells pre-incubated with KN-92. PDE4 inhibition potentiated the effect of Iso on CaT amplitude and SS, which were additionally increased by 87.7 ± 23.5% and 91.3 ± 7.0%, respectively, upon Iso + Ro (P < 0.001 vs. Iso, Figure 4B). Decay times of Ca^{2+} and contraction twitches were similarly decreased by Iso and further accelerated by Ro in cells pre-incubated with KN-93 or KN-92 (Figure 4C). Interestingly, although KN-93 did not prevent the inotropic and lusitropic effects of the PDE4 inhibitor, it reduced the number of cells exhibiting SCWs by 71.6 ± 12.0% (Figure 4D, P < 0.05 vs. KN-92). KN-93 also prevented the increase in diastolic Ca^{2+} levels observed in cells pre-incubated with KN-92 (P < 0.05 vs. Ctrl, Figure 4D and Supplementary material online, Table S3). Finally, pre-incubation of the cardiomyocytes with 100 nM of the autacamtide-2-related inhibitory peptide (AIP), a highly potent and specific substrate competitive inhibitor of CaMKII, recapitulated the inhibitory effects of KN-93 on the pro-arrhythmogenic Ca^{2+} events (Supplementary material online, Figure S4 and Table S3). The relative contribution of PKA and CaMKII to the setting of the SR Ca^{2+} leak and the increase of the SR Ca^{2+} load promoted by PDE4 inhibition were then assessed using the same protocol, as described in Figure 2. As shown in Figure 5, H-89 abolished the SR Ca^{2+} leak and decreased the SR Ca^{2+} load measured upon Iso + Ro by 47.1 ± 3.0% (P < 0.001, Figure 5B and C). Fractional release was drastically diminished by PKA inhibition from 69 ± 3% in Iso + Ro alone to...
37 ± 2% in the presence of H-89 (P < 0.001, Figure 5D). Similar to H-89, CaMKII inhibition with KN-93 drastically reduced the SR Ca\(^{2+}\) leak by 85.4 ± 10.1% (P < 0.001 vs. KN-92, Figure 5B). However, SR Ca\(^{2+}\) load was slightly increased in KN-93 (Figure 5C), and consequently, fractional Ca\(^{2+}\) release was decreased (Figure 5D, P < 0.01 vs. KN-92).

### 3.4 The exchange protein directly activated by cAMP Epac2 participates to the arrhythmias induced by PDE4 inhibition

To precise the signalling pathway leading to CaMKII activation upon PDE4 inhibition, we pre-incubated the cardiomyocytes with either...
10 μM CE3F4 to inhibit Epac1 or 5 μM ESI-05 to inhibit Epac2 (Figure 6A). None of the two Epac inhibitors affected the amplitude of CaT or SS under different experimental conditions (Figure 6B and C and Supplementary material online, Table S4). However, although the occurrence of SCWs was not impacted by Epac1 inhibition (Figure 6D), ESI-05 diminished SCWs occurrence in Iso + Ro by 41.2 ± 8.8% (Figure 6E, P < 0.05 vs. vehicle) and decreased SR Ca2+ leak by 32%, whereas neither SR Ca2+ load nor fractional release was affected by Epac2 inhibition (Supplementary material online, Figure S5).
slightly increased the SR Ca\(^{2+}\) level, and this was largely prevented by SR Ca\(^{2+}\) leakage promotion via PKA and CaMKII 

PDE inhibitors evoke arrhythmias via CaMKII

The aforementioned results clearly identify CaMKII as a mediator of the pro-arrhythmic effects elicited by PDE4 inhibition. In order to know whether this mechanism is specific to PDE4, we studied the effects of PDE3 inhibition on SR Ca\(^{2+}\) homeostasis (Figure 7). As expected, PDE3 inhibition with cilostamide (Cil, 1 \(\mu\)M) enhanced the positive inotropic effects of Iso (data not shown, but see Mika et al.\(^{29}\)). Cil potentiated SR Ca\(^{2+}\) leak by 52.8 ± 15.7% and SR Ca\(^{2+}\) load by 23.0 ± 6.8% (Figure 7B, \(P < 0.05\) vs. Iso) and significantly promoted fractional release (Figure 7B, \(P < 0.01\) vs. Iso). To assess whether CaMKII inhibition prevents the arrhythmogenic SR Ca\(^{2+}\) leak induced by Iso + Cil, cells were preincubated with KN-93 or KN-92. CaMKII inhibition did not modify the stimulatory effect of Iso + Cil on CaT amplitude and SS (Figure 7C), but largely prevented SR Ca\(^{2+}\) leak (Figure 7D, \(P < 0.001\) vs. KN-92) and slightly increased the SR Ca\(^{2+}\) load, and these effects were accompanied with decreased fractional release (Figure 7D, \(P < 0.001\) vs. KN-92).

3.5 CaMKII inhibition prevents the SR Ca\(^{2+}\) leak promoted by PDE3 inhibition upon \(\beta\)-AR stimulation

The aforementioned results clearly identify CaMKII as a mediator of the pro-arrhythmic effects elicited by PDE4 inhibition. In order to know whether this mechanism is specific to PDE4, we studied the effects of PDE3 inhibition on SR Ca\(^{2+}\) homeostasis (Figure 7). As expected, PDE3 inhibition with cilostamide (Cil, 1 \(\mu\)M) enhanced the positive inotropic effects of Iso (data not shown, but see Mika et al.\(^{29}\)). Cil potentiated SR Ca\(^{2+}\) leak by 52.8 ± 15.7% and SR Ca\(^{2+}\) load by 23.0 ± 6.8% (Figure 7B, \(P < 0.05\) vs. Iso) and significantly promoted fractional release (Figure 7B, \(P < 0.01\) vs. Iso). To assess whether CaMKII inhibition prevents the arrhythmogenic SR Ca\(^{2+}\) leak induced by Iso + Cil, cells were preincubated with KN-93 or KN-92. CaMKII inhibition did not modify the stimulatory effect of Iso + Cil on CaT amplitude and SS (Figure 7C), but largely prevented SR Ca\(^{2+}\) leak (Figure 7D, \(P < 0.001\) vs. KN-92) and slightly increased the SR Ca\(^{2+}\) load, and these effects were accompanied with decreased fractional release (Figure 7D, \(P < 0.001\) vs. KN-92).

4. Discussion

PDE3 and PDE4 are the main PDE families degrading cAMP generated upon \(\beta\)-AR stimulation in the heart. Cardiotonic drugs such as milrinone target these enzymes and more specifically the PDE3 family to improve cardiac output in HF or after surgery for congenital cardiac diseases.\(^{18}\) By elevating cAMP, these treatments have beneficial haemodynamic actions but promote sudden cardiac death due to arrhythmias, which seriously compromise their use.\(^{18,20,34}\) PDE4 downregulation also enhances ECC and abnormal pro-arrhythmic spontaneous Ca\(^{2+}\) release events.\(^{22,24,25}\) This study provides new insights into the underlying cellular mechanisms of these electrophysiological perturbations associated with the use of PDE inhibitors. We show that upon \(\beta\)-AR stimulation, the positive inotropic effects of PDE4 inhibition are accompanied by increased SR Ca\(^{2+}\) load and leak, leading to elevated diastolic Ca\(^{2+}\) levels and increased occurrence of SCWs. We demonstrate that PDE4 inhibition exerts positive inotropic effects via PKA but induces a pro-arrhythmogenic SR Ca\(^{2+}\) leak via both PKA and CaMKII, the latter being activated via the classical cAMP/PKA pathway and to some extent via Epac2. Finally, we show that CaMKII inhibition can also prevent the SR Ca\(^{2+}\) leak promoted by PDE3 inhibitors. Thus, our results show that inhibiting CaMKII could prevent the pro-arrhythmic effects of PDE inhibitors while preserving their beneficial inotropic effects.

PDE3 or PDE4 inhibition promotes the inotropic effects of a nonmaximal \(\beta\)-AR stimulation by increasing PKA phosphorylation of LTCC, RyR2, PLB, and contractile proteins.\(^{29}\) The increase in CaT amplitude is consistent with an enhanced Ca\(^{2+}\) entry via the LTCC\(^{35}\) and the increased SR Ca\(^{2+}\) load.\(^{36}\) PLB phosphorylation by PKA at Ser16,
which is essential for β-AR stimulation of ECC.26 is potentiated by PDE4 inhibition (as shown in this study) as well as by PDE3 inhibition.29 PKA phosphorylation of RyR2 at Ser2808, even though controversial,38 has been shown to promote its sensitivity to Ca2+39 Altogether, these mechanisms can explain the increased SR Ca2+ load and fractional release observed upon PDE4 and PDE3 inhibition. According to study, H-89 at 10 μM, the concentration required to inhibit most of the PKA activity in ARVMs under our experimental conditions (Supplementary material online, Figure S2), prevented all the inotropic and lusitropic effects of PDE4 inhibition. However, H-89 at this concentration can also inhibit SERCA activity.40 This could explain the slower relaxation of CaT observed in H-89, and by limiting the SR Ca2+ load, it could have precluded the PKA-independent stimulatory effects of Epac on ECC reported earlier.11 Nonetheless, overexpression of PKI, a more specific PKA inhibitor but equally effective to inhibit the kinase in cardiomyocytes,40 consistently abolished the inotropic and lusitropic effects of β-AR stimulation with or without concomitant PDE4 inhibition (Supplementary material online, Figure S3). These results and the lack of effect of Epac and CaMKII inhibitors on CaT amplitude and shortening shown in Figures 4 and 6 further demonstrate that PKA is responsible for the cardiotonic effects of PDE4 inhibition, in accordance with the preserved β-AR stimulation of ECC observed in mice invalidated for Epac10 or CaMKII.41 Upon submaximal β-AR stimulation, PDE4 inhibition increases diastolic Ca2+ levels and the occurrence of SCWs, suggesting SR Ca2+ leak via RyR2.8 It is known that upon β-AR stimulation, not only PKA but also CaMKII is activated to promote SR Ca2+ leak.3 Alongside PKA and in agreement with a recent report,28 CaMKII is also activated upon PDE4 inhibition as demonstrated by PLB and RyR2 phosphorylation on Thr17 and Ser2814, respectively (Supplementary material online, Figure S1). Unlike what has been reported for the SR Ca2+ leak promoted by Iso in rabbit cardiomyocytes,6–8 inhibition of PKA abolished the increase in diastolic Ca2+ levels, the SR Ca2+ leak, and SCWs, demonstrating that PKA is critical for the pro-arrhythmic effects of the PDE4 inhibitor. This discrepancy could be due either to species differences or to the concentration of H-89 used to block PKA. Indeed, H-89 was used at 1 μM concentration in previous studies,6–8 which was clearly insufficient in our hands to fully block PKA activity in intact cells (Supplementary material online, Figure S2). Interestingly, CaMKII

Figure 6 Effect of Epac1 and Epac2 inhibitors on the ECC and the occurrence of SCWs promoted by PDE4 inhibition upon β-AR stimulation. (A) Representative traces of CaT and SCWs recorded in Fura-2-loaded ARVMs pre-incubated with vehicle (dimethyl sulfoxide), the Epac1 inhibitor CE3F4 (10 μM), or the Epac2 inhibitor ESI-05 (5 μM) and challenged with Iso (1 nM) + Ro (10 μM). (B) Mean amplitude (± SEM) of CaT (upper graph) and SS (lower graph) recorded upon Ctrl, Iso, and Iso + Ro from cells treated with either CE3F4 (black bars, n = 16 cells, six rats) or vehicle (white bars, n = 18 cells, six rats). (C) Same parameters from cells pre-incubated with ESI-05 (black bars, n = 15 cells, six rats) or vehicle (white bars, n = 22 cells, six rats). Average number of SCWs (± SEM) occurring during a 10 s pause in pacing under Ctrl, Iso, or Iso + Ro conditions from cells treated with CE3F4 or ESI-05 or their respective vehicles are presented in (D) and (E), respectively. *P < 0.01 (vs. Ctrl); **P < 0.01 (Iso vs. Iso + Ro); and ***P < 0.001 (vehicle vs. ESI-05). ANOVA followed by a Tukey test for CaT and SS or by a Dunn test to analyse the occurrence of SCWs.
Figure 7  Effect of CaMKII inhibition on ECC and SR Ca\textsuperscript{2+} leak and load promoted by PDE3 inhibition upon β-ARs in ARVMs. (A) Representative traces of CaT and SR Ca\textsuperscript{2+} leak and load measurements obtained in Fura-2 loaded ARVMs paced at 1 Hz, under basal conditions (Ctrl), upon β-AR stimulation with Iso (1 nM) alone, or with concomitant PDE3 inhibition by cilostamide (Cil, 1 μM). Tetracaine (1 mM) was used to measure SR Ca\textsuperscript{2+} leak, and caffeine (10 mM) was used to measure SR Ca\textsuperscript{2+} load. (B) Mean amplitude (± SEM) of SR Ca\textsuperscript{2+} leak (left panel), SR Ca\textsuperscript{2+} load (middle panel), and fractional release (right panel) recorded in Ctrl (white bars), Iso (grey bars), and Iso + Cil (black bars). (C) Mean amplitude (± SEM) of CaT (left panel) and SS (right panel) in Iso + Cil with 10 μM KN-92 (white bars) or 10 μM KN-93 (black bars). (D) Mean amplitude (± SEM) of SR Ca\textsuperscript{2+} leak (left panel), SR Ca\textsuperscript{2+} load (middle panel), and fractional release (right panel) measured in Iso + Cil conditions in the presence of 10 μM KN-92 (white bars) or 10 μM KN-93 (black bars). Number of cells obtained from three to four rats is indicated inside each bar representing the mean. Statistical significance is indicated as: *P < 0.05; ***P < 0.001 (vs. Ctrl); #P < 0.05; ##P < 0.01 (Iso vs. Iso + Cil); and $$$P < 0.001 (Iso + Cil + KN-92 vs. Iso + Cil + KN-93) (ANOVA followed by a Tukey post hoc test).
inhibition diminished the number of cells exhibiting SCWs and drastically
blunted the SR Ca\(^{2+}\) leak. Furthermore, the increase in fractional
release induced by Iso and concomitant PDE4 inhibition was abolished
by H-89 and decreased by KN-93, suggesting that both kinases partici-
pate in the Ca\(^{2+}\) sensitization of RyR2. Whether PKA phosphoryla-
tion of RyR2 participates in this process remains controversial\(^{38,42}\) and
cannot be ruled out from our experiments. Nonetheless, our results cor-
rborate that both kinases must be activated to increase RyR2 sensi-
tivity, as recently proposed.\(^{43}\) Furthermore, because PDE3 or
PDE4 inhibition increases SR Ca\(^{2+}\) load via PKA phosphorylation
of PLB, this could by itself explain the RyR2 sensitization to Ca\(^{2+}\),
given that luminal Ca\(^{2+}\) determines RyR2 open probability.\(^{34}\) In
addition, promoted SERCA activity will enable sufficient SR Ca\(^{2+}\)
levels close to the threshold necessary to generate Ca\(^{2+}\) waves, as demonstrated
previously.\(^{45}\) Thus, PKA is the primary mediator of SR Ca\(^{2+}\) leak
promoted by PDE4 inhibition, because it increases the SR Ca\(^{2+}\) load
required to trigger Ca\(^{2+}\) waves.\(^{45}\) Most likely by increasing the L-type Ca\(^{2+}\)
current and SERCA2 activity via PLB phosphorylation. Indeed, PKA leads to CaMKII activation by the classical Ca\(^{2+}\)/CaM
pathway, a phenomenon which will increase RyR2 phosphorylation by CaMKII which alongside PKA contributes to in-
crease RyR2 sensitivity under β-AR stimulation.\(^{03}\) CaMKII inhibition
limits SR Ca\(^{2+}\) leak upon β-AR stimulation and concomitant PDE4 in-
hibition without affecting the SR Ca\(^{2+}\) content. This could be explained by
increased Ca\(^{2+}\) sensitivity of RyR2 due to the increased phosphory-
atation at Ser281446 as observed here, whereas PKA phosphorylation
of ESI-05. Alternatively, it may suggest that CaMKII activation via Epac2 is minor when compared with the clas-
sical PKA-mediated cytosolic Ca\(^{2+}\) elevation. Of note, ESI-05 partially
diminished the occurrence of SCWs in Iso + Ro, but not the few
events observed under non-maximal β-AR stimulation alone. This could
be explained by the higher cAMP levels required for Epac activation
compared to PKA activation.\(^{47}\) Whether activation of Epac2 is under
the control of PDE3 remains to be determined. However, CaMKII in-
hibition prevented the increased SR Ca\(^{2+}\) leak induced by cistostamide,
demonstrating that this kinase participates in the arrhythmias evoked
by PDE3 inhibitors.

To conclude, our work provides new insights into the cellular me-
chanisms underlying the arrhythmias observed upon PDE3 and PDE4
inhibition. It demonstrates that PDE3 and PDE4 inhibitors exert in-
tropic effects via PKA by increasing SR Ca\(^{2+}\) load, but shows for the
first time that they promote diastolic pro-arrhythmic Ca\(^{2+}\) waves
linked to an SR Ca\(^{2+}\) leak via both PKA and CaMKII. Interestingly,
CaMKII inhibition prevented arrhythmias but did not alter the inotropic
effects of the PDE inhibitors. Additional experiments are needed to
evaluate whether the present results may be transposable to a patho-
logical state, particularly in the context of HF in which the β-AR/cAMP
pathway is altered.\(^{4}\) However, in the context of acute and decompens-
sated HF in which Epac\(^{39}\) and CaMKII\(^{4}\) are upregulated, PDE inhibitors
have been shown to exert beneficial effects on haemodynamics. Fur-
thermore, CaMKII is likely to contribute to the pro-arrhythmic effects
of these drugs in HF, as its activity is increased by elevated cytosolic
Ca\(^{2+}\) levels and oxidative stress evoked by the pathological activation
of β-AR and renin–angiotensin–aldosterone pathways.\(^{49}\) Interestingly,
CaMKII inhibition can also prevent apoptosis,\(^{50}\) another adverse conse-
quence of PDE inhibition.\(^{20,51}\) Altogether, our results suggest the
potential use of CaMKII inhibitors in adjunct to PDE inhibitors to
counteract their side effects while preserving their cardio-
tonic properties.

**Supplementary material**

Supplementary Material is available at Cardiovascular Research online.

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