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# Early effects of Epac depend on the fine-tuning of the sarcoplasmic reticulum $Ca^{2+}$ handling in cardiomyocytes

N. Lezcano[a], J.I.E. Mariángelo[a], L. Vittone[a], X.H.T. Wehrens[b], M. Said[a], C. Mundiña-Weilenmann[a,*]

[a] Centro de Investigaciones Cardiovasculares, CCT-CONICET La Plata, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, Argentina
[b] Cardiovascular Research Institute, Baylor College of Medicine, Houston, TX, United States

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ABSTRACT

In cardiac muscle, signaling through cAMP governs many fundamental cellular functions, including contractility, relaxation and automatism. cAMP cascade leads to the activation of the classic protein kinase A but also to the stimulation of the recently discovered exchange protein directly activated by cAMP (Epac). The role of Epac in the regulation of intracellular $Ca^{2+}$ homeostasis and contractility in cardiac myocytes is still matter of debate. In this study we showed that the selective Epac activator, 8-(4-chloro-phenylthio)-2′-*O*-methyladenosine-3′, 5′-cyclic monophosphate (8-CPT), produced a positive inotropic effect when adult rat cardiac myocytes were stabilized at low $[Ca^{2+}]_o$ (0.5 mM), no changes at 1 mM $[Ca^{2+}]_o$ and a negative inotropic effect when $[Ca^{2+}]_o$ was increased to 1.8 mM. These effects were associated to parallel variations in sarcoplasmic reticulum (SR) $Ca^{2+}$ content. At all $[Ca^{2+}]_o$ studied, 8-CPT induced an increase in $Ca^{2+}$ spark frequency and enhanced CaMKII autophosphorylation and the CaMKII-dependent phosphorylation of SR proteins: phospholamban (PLN, at Thr17 site) and ryanodine receptor (RyR2, at Ser2814 site). We used transgenic mice lacking PLN CaMKII phosphorylation site (PLN-DM) and knock-in mice with an inactivated CaMKII site S2814 on RyR2 (RyR2-S2814A) to investigate the involvement of these processes in the effects of Epac stimulation. In PLN-DM mice, 8-CPT failed to induce the positive inotropic effect at low $[Ca^{2+}]_o$ and RyR2-S2814A mice showed no propensity to arrhythmic events when compared to wild type mice myocytes. We conclude that stimulation of Epac proteins could have either beneficial or deleterious effects depending on the steady-state $Ca^{2+}$ levels at which the myocyte is functioning, favoring the prevailing mechanism of SR $Ca^{2+}$ handling (uptake vs. leak) in the different situations.

## 1. Introduction

cAMP is a universal second messenger that plays a central role in the regulation of cardiac contractility. In the last years, it has become recognized that along with the cAMP effector protein kinase A (PKA), the exchange protein directly activated by cAMP (Epac) participates in many cAMP-controlled processes of heart function. Among them, activation of Epac has been involved in the regulation of $Ca^{2+}$ homeostasis in cardiac myocytes, $Ca^{2+}$ myofilament sensitivity, gap junction formation, arrhythmogenesis, apoptosis, autophagy, hypertrophy, vascular integrity and cardiac fibrosis [1,2].

The Epac protein family is composed of Epac1 and Epac2, which act as guanine-nucleotide exchange factors for the small G proteins Rap1 and Rap2, in a PKA-independent manner. In mouse and human hearts, Epac1 is the most abundant isoform [3,4] and its expression is developmentally regulated, with the Epac1/Epac2 mRNA ratio decreasing in adulthood [4].

Several studies in isolated cardiac myocytes have shown that stimulation of Epac by the selective activator, 8-(4-chloro-phenylthio)-2′-*O*-methyladenosine-3′, 5′-cyclic monophosphate (8-CPT), increased the activity of the $Ca^{2+}$ and calmodulin-dependent protein kinase II (CaMKII) and the phosphorylation of two sarcoplasmic reticulum (SR) targets, the $Ca^{2+}$ release channel (RyR2) and the $Ca^{2+}$ pump (SERCA2a) regulator, phospholamban (PLN) [3,5–8]. Moreover, at the level of the myofibrils, 8-CPT enhanced the CaMKII-dependent phosphorylation of myosin-binding protein C (MyBPC) and troponin I (TnI) [9]. Even though CaMKII appears as a clear downstream signal of Epac, the pathway that leads to its activation is still debated. The Epac-mediated effects have been shown to require the presence of ε isoform of phospholipase C (PLCε) [5,7] and the resultant increase in cytosolic $Ca^{2+}$ triggered by IP3 as well as the diacylglycerol-activated PKCε, have been implicated in the stimulation of CaMKII under different

* Corresponding author at: Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, 60 y 120, 1900 La Plata, Argentina.
*E-mail address:* cmundweil@med.unlp.edu.ar (C. Mundiña-Weilenmann).

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experimental conditions (5, 7, 10). Additionally, it has recently been reported a nitric oxide synthase and phosphoinositide 3-kinase dependent activation of CaMKII during Epac stimulation [8].

The role of Epac in the regulation of intracellular $Ca^{2+}$ homeostasis and contractility is still matter of debate. In rat adult cardiomyocytes, acute Epac stimulation decreased the amplitude of $Ca^{2+}$ transients [6,9,10] with either no changes [6] or increments [9] in cell shortening, suggesting an enhancement of myofilament $Ca^{2+}$ sensitivity. This was confirmed in the latter study and supported by the finding of the increased CaMKII-phosphorylation of MyBPC and TnI [9]. The diminished $Ca^{2+}$ transient was paralleled by a decrease in the amount of $Ca^{2+}$ stored in the SR, attributed to the increased SR $Ca^{2+}$ leak induced by the CaMKII-dependent phosphorylation of RyR2 [6]. In contrast to rat myocytes, an increase [5,7] or no change in $Ca^{2+}$ transient [11] were detected in mice myocytes after acute stimulation of Epac. As in the rat, 8-CPT induced an enhancement of the CaMKII-dependent phosphorylation of RyR2 and PLN [7]. Furthermore, the Epac-specific agonist caused spontaneous triggered activity in intact perfused murine hearts, associated with increased incidence of spontaneous $Ca^{2+}$ transients and propensity to the generation of $Ca^{2+}$ waves at the myocyte level [11]. Such arrhythmogenic features were also observed in rat myocytes but after sustained Epac activation [12]. In this case, rat myocytes showed an increase in $Ca^{2+}$ transient, cell shortening and SR $Ca^{2+}$ content, favored by enhanced $Ca^{2+}$ influx through the L-type $Ca^{2+}$ channels. The development of KO mice did not help to clarify the specific involvement of Epac in cardiac contractile behavior. Pereira et al. 2013 [13] showed unaltered basal cardiac function and $Ca^{2+}$ handling in KO mice of either Epac1 or Epac2 and double KO mice. Moreover Epac2 and not Epac1, was shown to be essential for 8-CPT-induced RyR2 activation, enhanced $Ca^{2+}$ leak and decreased $Ca^{2+}$ transient [13]. However, Okumura et al. 2014 [14] found that loss of Epac1 decreased basal cardiac contractility, reduced $Ca^{2+}$ transient and diminished SR $Ca^{2+}$ storage. Overall, the effects of Epac in intracellular $Ca^{2+}$ handling and contractility remain controversial. The apparent discrepancy may depend on experimental conditions. For instance, genetic background in the KO models, acute vs. chronic effects of 8-CPT, species and/or different extracellular $Ca^{2+}$ ($[Ca^{2+}]_o$) to which the myocytes are exposed. Related to the latter, it is important to consider that $Ca^{2+}$ supply to the cell alters intracellular $Ca^{2+}$, dynamically adjusting the balance between SR $Ca^{2+}$ uptake and leak.

The aim of the present study was to elucidate if the acute effects of Epac stimulation depend on the SR $Ca^{2+}$ handling state. In determining this, we varied $[Ca^{2+}]_o$ in order to shift the SR balance from net $Ca^{2+}$ accumulation to net $Ca^{2+}$ release and we focused on the relevance of CaMKII-dependent phosphorylation of PLN and RyR2 in the response to Epac stimulation, through the use of transgenic mice with non-phosphorylatable CaMKII sites.

## 2. Materials and methods

### 2.1. Animals

The experiments were performed in male Wistar rats (200–300 g body weight), mice (25–30 g) with genetic ablation of the CaMKII phosphorylatable site on RyR2 (RyR2-S2814A knock-in) [15] and mice expressing a mutant PLN in which both phosphorylatable residues (Ser16 and Thr17) were replaced by Ala (PLN-DM) (MMRRC, University of Missouri/Harlam, Mouse Regional Resource Center, NCRR, NIH) [16]. Transgenic mice were backcrossed to the C57BL/6 for over 10 generations. Age-matched wild type C57BL/6 mice (WT) served as controls. Animals were maintained in accordance with the Institutional Animal Care and Use Committee (IACUC) of the School of Medicine, National University of La Plata, Argentina (Nro T05022014) conforming to the Guide for the Care and Use of Laboratory Animals (NIH, 2011).

### 2.2. Myocyte isolation

Myocytes were isolated by enzymatic digestion as previously described [17]. Briefly, after reached phase III anesthesia verified by the loss of pedal withdrawal reflex (intraperitoneal injection of Ketamine/Diazepam (70 mg/kg/5 mg/kg for rat and 100 mg/kg 5 mg/kg for mice) central thoracotomy were performed. The hearts were anticoagulated with heparin (2.5 units/g body weight) excised and mounted in a Langendorff apparatus. They were then retrogradly perfused at 37 °C at a constant perfusion pressure of 80–90 mm Hg with Hepes Buffer Solution (HBS) of the following composition (mM): 146.2 NaCl, 4.7 KCl, 1.0 $CaCl_2$, 10.0 Hepes, 0.35 $NaH_2PO_4$, 1.05 $MgSO_4$, 10.0 glucose (pH adjusted to 7.4 with NaOH). The solution was continuously bubbled with 100% $O_2$. After stabilization period of 4 min, the perfusion was switched to a nominally $Ca^{2+}$-free HBS solution for 6 min. Hearts were then recirculated with collagenase (118 U $mL^{-1}$) 0.1 mg $mL^{-1}$ pronase and 1% bovine serum albumin (BSA), in HBS containing 50 μM $CaCl_2$. Perfusion continued until the hearts became flaccid (15–25 min). They were then removed from the perfusion apparatus by cutting at the atria-ventricular junction. The desegregated myocytes were separated from the undigested tissue and rinsed several times with a HBS solution containing 1% BSA and 500 μM $CaCl_2$. Ventricular myocytes were dispersed mechanically and filtered through a nylon mesh and allowed to sediment for 10 min. The sedimentation is repeated three times every 10 min, while $[Ca^{2+}]$ (mM) was increased stepwise, from 0.125 to 0.25 through 1 mM $[Ca^{2+}]_o$ (rat) or 1.8 (mice). Only rod-shaped myocytes with clear and distinct striations and an obvious marked shortening and relaxation on stimulation were used.

### 2.3. Myocyte shortening and $[Ca^{2+}]_i$ measurements

Isolated myocytes were loaded with Fura-2/AM (2 μmol/L for 15 min). Residual extracellular dye was removed by centrifugation and the pellet was washed three times. $[Ca^{2+}]_i$ was measured with an epifluorescence system (Ion Optix, Milton, MA, USA). Briefly, dye-loaded cells were placed in a chamber on the stage of an inverted microscope (Nikon.TE 2000-U) and continuously superfused with a HBS at a constant flow of 1 mL/min. Experiments were performed at room temperature (20–22 °C) and myocytes were stimulated via two-platinum electrodes on either side of the bath at 1 Hz. The ratio of the Fura-2 fluorescence (510 nm) obtained after exciting the dye at 340 and 380 nm was taken as an index of $[Ca^{2+}]_i$. Resting sarcomere length and cell shortening were measured by a video-based motion detector (Crescent electronics, UT, USA). Myocytes were equilibrated in HBS at different $[Ca^{2+}]_o$ and measurements were performed before and after the addition of 8-(4-Chlorophenylthio)-2′-*O*-methyladenosine- 3′, 5′-cyclic monophosphate (8-CPT, 10 μM) (Biolog). Different $[Ca^{2+}]_o$ were selected from experiments in which the influence of $[Ca^{2+}]_o$ on 8-CPT response was evaluated. For rat myocytes, 0.5 mM, 1 mM and 1.8 mM $[Ca^{2+}]_o$ were chosen because at these concentrations 8-CPT showed a positive, unchanged or negative inotropic effect respectively. For mice myocytes, 1.8 mM and 2.5 mM $[Ca^{2+}]_o$ were the concentrations at which 8-CPT showed a positive and no change in the inotropic response respectively. Fluorescence and cell shortening data were stored for off-line analysis (ION WIZARD fluorescence analysis software). $Ca^{2+}$ transients were analyzed as the mean value over a 10–12 records for each cell. SR $Ca^{2+}$ content was determined by rapidly switching from the HBS to one of the same pH, containing 25 mM caffeine to cause SR $Ca^{2+}$ release. Myocytes showing two or more spontaneous non-stimulated contractions and $Ca^{2+}$ transients were considered arrhythmic.

### 2.4. $Ca^{2+}$ sparks measurement

Rat ventricular myocytes were loaded with 10 μM Fluo-4-AM (Invitrogen) in HBS containing 1.0 mM $[Ca^{2+}]_o$ for 20 min at room temperature, and mounted in a small chamber placed into an inverted

microscope equipped with a 63 × objective as previously described [18]. After stabilization (usually 3–5 min), confocal line-scanning (512 × 512 pixels and 4.3 ms per line) was performed along the longitudinal axis of cells (avoiding nuclei), using the Zeiss LSM 210 confocal system in quiescent cells. The Fluo-4 loaded myocytes were excited using the 488 nm argon laser and the fluorescence emission was recorded at 500–550 nm. $Ca^{2+}$ sparks were measured using the 'Sparkmaster' plugin for ImageJ. A $Ca^{2+}$ wave was defined as a continuous wave front in the line scan image visualized as a robust fluorescent line that propagates across the full width of the myocyte without breaking.

### 2.5. Electrophoresis and western blot analysis

To determine the phosphorylation state of different proteins in response to Epac stimulation, rat isolated myocytes were plated on 35-mm tissue culture dishes, stimulated at 1 Hz and treated with or without 10 μM 8-CPT (Biolog) for 5–10 min in HBS containing 0.5; 1 and 1.8 mM $[Ca^{2+}]_o$. When PKC inhibitor (calphostine C) was used, it was pre-incubated for 20 min before the addition of 8-CPT. Treatment was stopped by immersing the dishes in liquid nitrogen. Cells were scraped off and centrifuged at 8000 × g for 5 min. The pellet was resuspended in lysis buffer containing (in mM): 20 β-glycerophosphate, 50 NaF, 1 $Na_4P_2O_7$; 2 $Na_3VO_4$; 2 EGTA; 2 EDTA; 1 μM okadaic acid; 1% Triton X-100, 1% SDS and complete protease inhibitor cocktail (Roche). After protein measurement by Bradford's method, samples were resolved in SDS-PAGE on either 6% gels (for RyR2) or 12% (w/v) gels (for the rest of the proteins), transferred to PVDF membranes and probed with antibodies raised against: Thr286-phosphorylated CaMKII (Abcam), Thr17-phosphorylated PLN (Badrilla), Ser16-phosphorylated PLN (Badrilla), total PLN (Abcam), Ser2814-phosphorylated RyR2 (Badrilla), Ser2808-phosphorylated RyR2 (Badrilla), total RyR2 (Thermo Scientific), Ser282-phosphorylated MyBP-C (Enzo Life Sciences) and MyBP-C (Santa Cruz Biotechnology) and GAPDH (Millipore) generally overnight. Membranes were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and developed using an enhanced chemiluminescence reagent (Millipore). The signals emitted were detected using Chemidoc Imaging System (Bio-Rad) and analyzed with ImageJ software (NIH, Bethesda, MD, USA). The results were expressed by normalizing the densitometry signal obtained by GAPDH, PLN or RyR2 as corresponding.

### 2.6. Statistical analysis

Data are express as mean ± SEM. Unpaired, paired Student *t*-test or ANOVA followed by The Newman-Keuls test were used for statistical comparisons when appropriate. The incidence of arrhythmias was analyzed using Fisher's exact probability test. Differences were considered significant at $p < 0.05$.

## 3. Results

### 3.1. Epac effects on contractility and intracellular $Ca^{2+}$ handling depend on $Ca^{2+}$ supply to the myocyte

To find an explanation for the contradictory results regarding the effects of Epac activation on contractility and intracellular $Ca^{2+}$ homeostasis, we simultaneously measured cell shortening and intracellular $Ca^{2+}$ transient in rat cardiomyocytes stimulated with the Epac selective activator, 8-CPT, at different $[Ca^{2+}]_o$. At 0.5 mM $[Ca^{2+}]_o$, 8-CPT elicited an increase in fractional sarcomere shortening and in steady-state twitch $Ca^{2+}$ transient amplitude (Fig. 1A–C). This positive inotropic effect was not observed at 1 mM $[Ca^{2+}]_o$ and turned into a negative inotropic effect when $[Ca^{2+}]_o$ was increased to 1.8 mM. Furthermore, only at low $[Ca^{2+}]_o$, exposure to 8-CPT produced a relaxant effect, evidenced by an acceleration of $Ca^{2+}$ transient decay and myocyte relengthening (Fig. 1D and E). The study of the kinetics of the sarcomere shortening and Tau for $Ca^{2+}$ transient support the Epac-induced inotropic and relaxant effects observed at the different $[Ca^{2+}]_o$ (Fig. 1S A and B). In an attempt to examine 8-CPT-induced changes in myofilament $Ca^{2+}$ sensitivity we plotted a phase-plane loop of sarcomere length vs. $Ca^{2+}$ transient before and after 8-CPT treatment. At each $[Ca^{2+}]_o$ explored, the relaxation phases in the absence and the presence of 8-CPT were superimposed suggesting no changes in the apparent myofilament $Ca^{2+}$ sensitivity (Fig. 2S).

To determine if the effects of 8-CPT on contractility and $Ca^{2+}$ transient were associated to changes in SR $Ca^{2+}$ load, we measured caffeine-induced $Ca^{2+}$ transients (Fig. 2A and B). The results showed increase, no change and decrease in SR $Ca^{2+}$ content at 0.5; 1 and 1.8 mM $[Ca^{2+}]_o$ respectively. Thus, 8-CPT-induced modifications in SR $Ca^{2+}$ load could account for the inotropic changes produced by the Epac activator. Additionally, 8-CPT did not modify the decay time of the caffeine-evoked $Ca^{2+}$ transient at any of the $[Ca^{2+}]_o$ studied, indicating that Epac stimulation did not alter the activity of the $Na^+$-$Ca^{2+}$ exchanger (NCX) (Table S1).

To evaluate the effect of 8-CPT on spontaneous SR $Ca^{2+}$ release events at the different $[Ca^{2+}]_o$, imaging studies were performed. Representative line-scan recordings (Fig. 2C) and bar graphs containing summary data (Fig. 2D) showed that 8-CPT enhanced the frequency of $Ca^{2+}$ sparks at all $[Ca^{2+}]_o$ studied with amplitude, duration and width of the $Ca^{2+}$ sparks similar among groups (Table 2S). This spontaneous SR $Ca^{2+}$ release was associated to enhanced diastolic $Ca^{2+}$ at 1 and 1.8 mM $[Ca^{2+}]_o$ (Fig. 1S C). Moreover, at 1.8 mM $[Ca^{2+}]_o$ the Epac agonist initiated self-propagating $Ca^{2+}$ waves which are the result of a substantial SR $Ca^{2+}$ leak. Given that $Ca^{2+}$ spark frequency is highly dependent on SR $Ca^{2+}$ content, we compared the data obtained in Fig. 2B and D. Only at 0.5 mM $[Ca^{2+}]_o$, the increased in $Ca^{2+}$ sparks induced by 8-CPT could be attributed to an increase in SR $Ca^{2+}$ load. At 1 and 1.8 mM $[Ca^{2+}]_o$, there was no relationship between spontaneous SR $Ca^{2+}$ release events and SR $Ca^{2+}$ content. In fact, at 1.8 mM $[Ca^{2+}]_o$, the Epac agonist maximally increased $Ca^{2+}$ spark frequency, despite a significantly diminished SR $Ca^{2+}$ content. These apparently contradictory results could be explained by alterations at the level of RyR2, which can lead to abnormal SR $Ca^{2+}$ spontaneous release independently on its $Ca^{2+}$ content.

Taken together, the present results demonstrated that the myocyte response to 8-CPT depends on $[Ca^{2+}]_o$ and suggest that the shift in the balance between SR $Ca^{2+}$ uptake and leak at the different $[Ca^{2+}]_o$, determines the outcome of the contractile behavior due to Epac activation.

### 3.2. Epac-induced CaMKII-dependent PLN and RyR2 phosphorylation at different $[Ca^{2+}]_o$

It has been previously shown that Epac controls CaMKII activity, in a PKA-independent manner, and enhances CaMKII target phosphorylation of Thr17 of PLN and Ser2814 of RyR2. Phosphorylation of these two SR substrates is known to increase SR $Ca^{2+}$ uptake and leak respectively. In order to investigate if PKC is responsible for CaMKII activation during Epac stimulation we performed experiments in the absence and presence of the PKC inhibitor, calphostin C (CC). Immunoblots and overall results of Fig. 3A and B confirmed that 8-CPT increased the phosphorylation of CaMKII at Thr286 (autophosphorylation), PLN at Thr17 and RyR2 at Ser2814. These increases were completely prevented by the application of CC, indicating that PKC was upstream of CaMKII in the Epac-stimulated signaling pathway in our experimental conditions.

The Epac activator failed to increase the phosphorylation of MyBPC, a phosphoprotein known to regulate myofilament $Ca^{2+}$ sensitivity (Fig. 3C). Moreover, 8-CPT did not increase the phosphorylation of Ser16 of PLN and Ser2808 of RyR2, two PKA phosphorylatable sites,

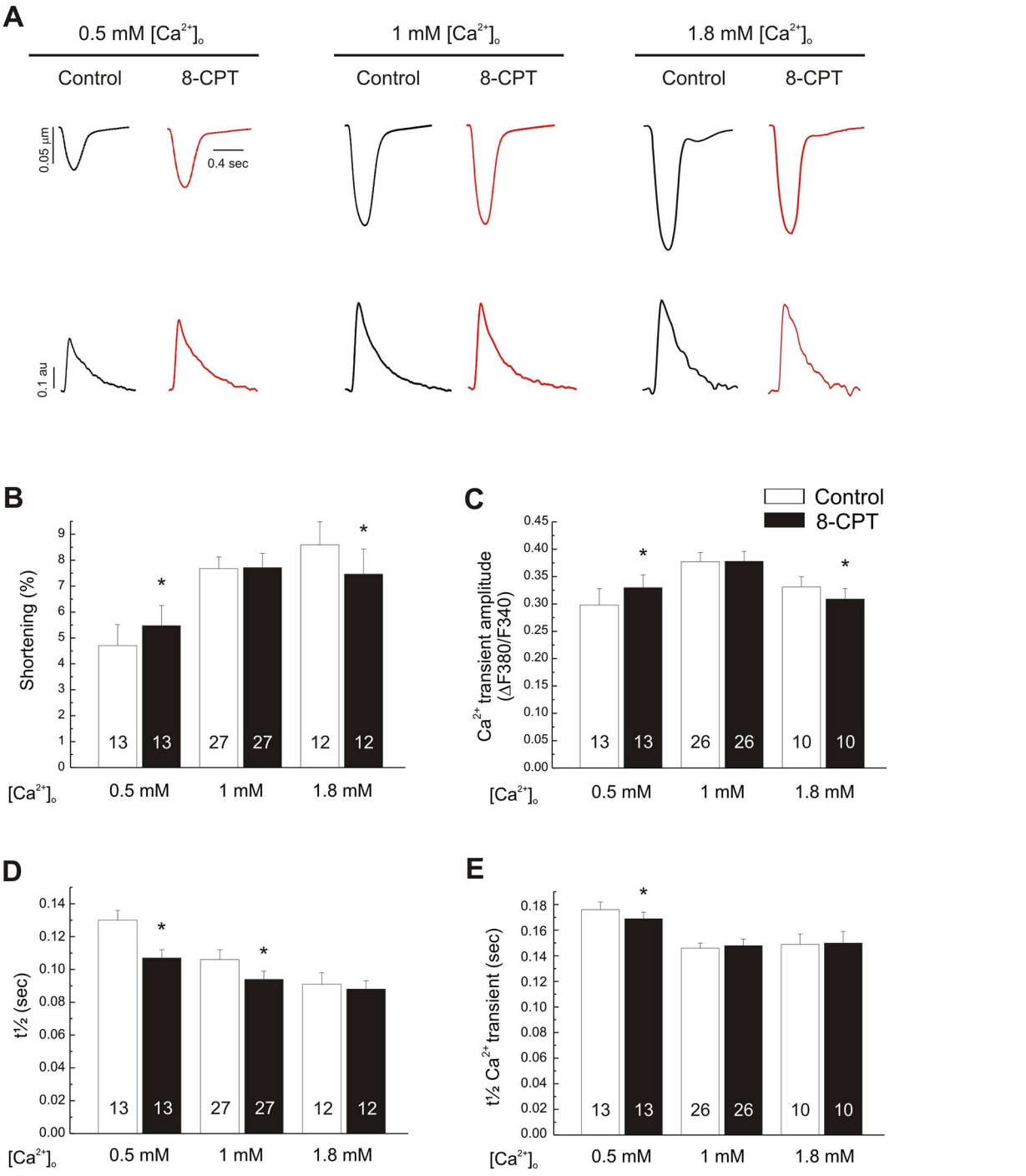


**Fig. 1.** Epac differentially modifies cell shortening, $[Ca^{2+}]_i$ transient and relaxation at different $[Ca^{2+}]_o$. A, representative simultaneous recordings of cell shortening (above) and $Ca^{2+}$ transient (below) in rat myocytes loaded with Fura-2 AM and field-stimulated at 1 Hz, before (black traces) and after (red traces) 10 µM 8-CPT application at 0.5, 1 and 1.8 mM $[Ca^{2+}]_o$; B, C, D and E, overall results of sarcomere cell shortening; $Ca^{2+}$ transient amplitude; time to 50% relaxation ($t_{1/2}$) of twitches and time to 50% decay of $Ca^{2+}$ transient ($t_{1/2}$ $Ca^{2+}$ transient) of rat myocytes before (white bars) and after (black bars) 10 µM 8-CPT. * $p < 0.05$ paired *t*-test vs. before 8-CPT stimulation at each $[Ca^{2+}]_o$. Numbers in bars indicate number of cells of at least 3 rats. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

confirming the selectivity of the drug for Epac (Fig. 3D and E).

To test whether differential CaMKII-dependent phosphorylations underlie different responses to Epac stimulation, we measured these phosphorylations in the absence and presence of 8-CPT at the three $[Ca^{2+}]_o$ used. In the absence of the drug, the increase in $[Ca^{2+}]_o$ from 0.5 to 1 mM significantly enhanced the phosphorylation of CaMKII and PLN, without increasing the phosphorylation of RyR2 (Fig. 3F–H). This could explain the net SR $Ca^{2+}$ gain (Fig. 2B) due to the PLN-induced increase in SR $Ca^{2+}$ uptake. Switching the $[Ca^{2+}]_o$ from 1 mM to 1.8 mM, further increased CaMKII and PLN phosphorylations and promoted the enhancement of RyR2 phosphorylation. This latter mechanism by facilitating SR $Ca^{2+}$ leak may be counteracting the effects of PLN phosphorylation and precluding the increase in SR $Ca^{2+}$ content (Fig. 2B). At all $[Ca^{2+}]_o$ studied, treatment of the cardiomyocytes with the Epac selective agonist increased CaMKII-dependent phosphorylations compared with control conditions in the absence of drug (Fig. 3C–E).

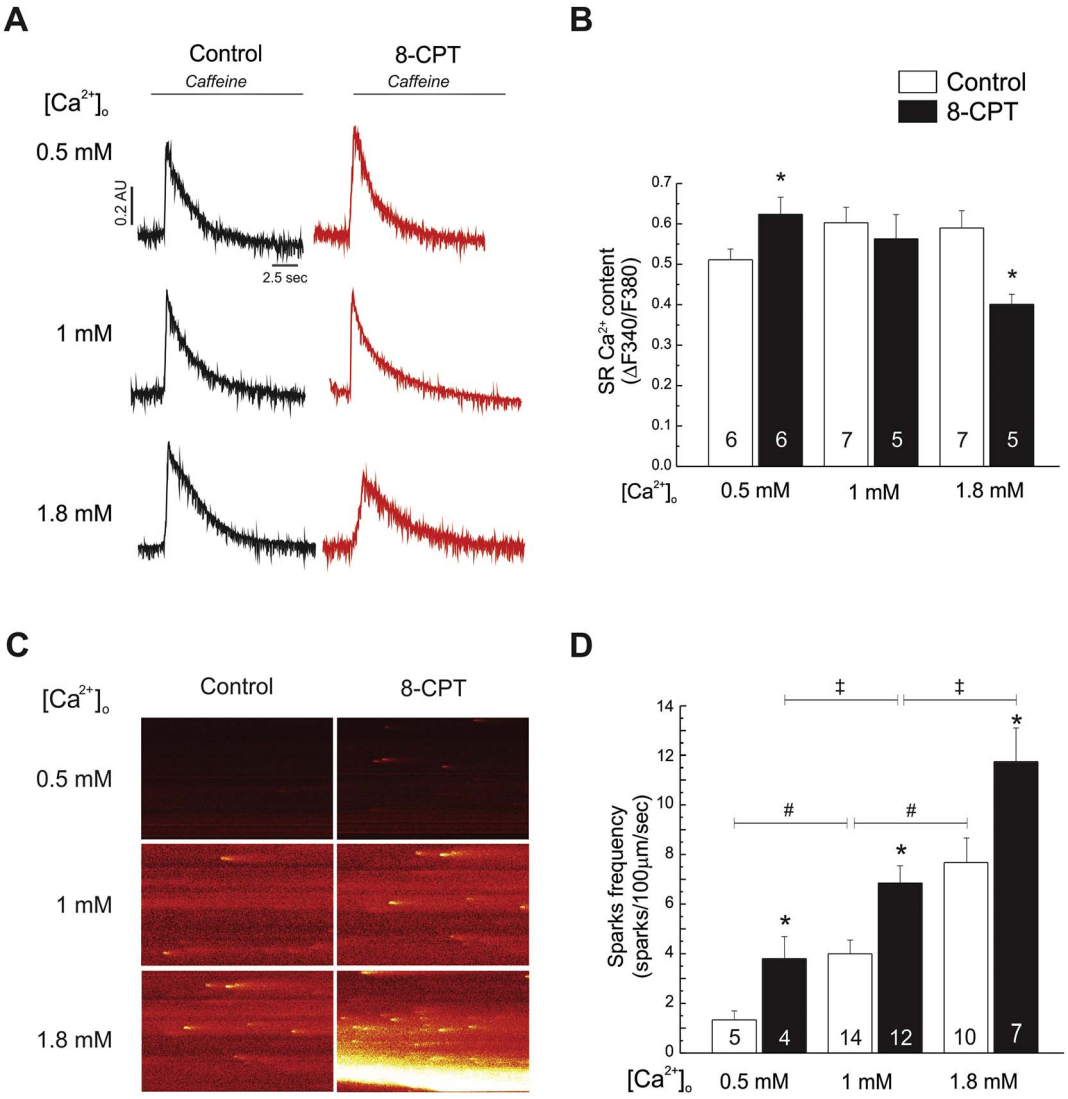


**Fig. 2.** Epac stimulation increases SR $Ca^{2+}$ content and $Ca^{2+}$ spark occurrence at different $[Ca^{2+}]_o$. A, typical records of caffeine-triggered $Ca^{2+}$ transients and B, averaged amplitude of these transients of rat myocytes loaded with Fura-2 AM in the absence and presence of 10 μM 8-CPT. C, representative line-scan images of spontaneous $Ca^{2+}$ sparks of myocytes loaded with Fluo-4 before (Control, left) and after treatment with 10 μM 8-CPT (right) at 0.5; 1 and 1.8 mM $[Ca^{2+}]_o$. D, bar graph showing the measured $Ca^{2+}$ spark frequency in control cells (white bars) and cells in the presence of 8-CPT (black bars) at different $[Ca^{2+}]_o$. Numbers in bars indicate number of cells from at least 3 rats. * $p < 0.05$ vs. control at each $[Ca^{2+}]_o$. # $p < 0.05$ vs. 1 mM $[Ca^{2+}]_o$ in the absence of drug.

### *3.3. Relative contribution of CaMKII-dependent PLN and RyR2 phosphorylations to the effects of Epac activation*

The findings presented in the previous section did not allow us to draw conclusions about the impact of CaMKII-dependent PLN (Thr17) and RyR2 (Ser2814) phosphorylations on the effects of Epac activation at different $[Ca^{2+}]_o$. To resolve this issue, we took advantage of transgenic mice models in which both sites were rendered non-phosphorylatable (PLN-DM and RyR2-S2814A). We first characterized the effects of Epac activation in wild type mouse myocytes, looking for experimental conditions (different $[Ca^{2+}]_o$) that provide a comparable basal contractile state to rat myocytes. When studying the impact of $[Ca^{2+}]_o$ on 8-CPT contractile response in mouse cardiomyocytes, a shift to the right with respect to the rat was observed. At 1.8 mM $[Ca^{2+}]_o$, 8-CPT elicited an increase in cell shortening and $Ca^{2+}$ transient amplitude and a decrease in the relaxation times (Fig. 4A–D). At 2.5 mM $[Ca^{2+}]_o$, the Epac activator failed to produce the inotropic and lusitropic effects. This behavior, similar to that observed in rat, indicates that differences in the response to Epac stimulation are not species-dependent. The 8-CPT studied effects, which appeared soon after drug exposure (1 min), remained stable for > 10 min at 1.8 mM $[Ca^{2+}]_o$ but at 2.5 mM $[Ca^{2+}]_o$, measurements of $[Ca^{2+}]_i$ transient and cell shortening were necessarily performed during the first 5 min of treatment because after this period, the Epac activator produced an increase in diastolic $Ca^{2+}$, a decrease in the initial resting length and promoted the appearance of arrhythmic events in all treated mouse myocytes (Fig. 5A and C).

We next tested the effects of Epac activation in myocytes from PLN-DM and RyR2-S2814A mice. The results showed that 8-CPT failed to induce positive inotropic and lusitropic effects in PLN-DM myocytes at 1.8 mM $[Ca^{2+}]_o$ (Fig. 4A–D). On the other hand, at 2.5 mM $[Ca^{2+}]_o$, 8-CPT-induced increments in resting length and diastolic $Ca^{2+}$ and the appearance of arrhythmias were completely abolished in RyR2-S2814A myocytes (Fig. 5B and C). The inotropic response of these mice to Epac activation did not differ from that observed in the wild type mouse myocytes (Fig. 3S).

Taken together these findings demonstrate that CaMKII-dependent PLN phosphorylation is a necessary step for the positive inotropic effect of 8-CPT at low $[Ca^{2+}]_o$ and that CaMKII-dependent RyR2 phosphorylation is responsible for the spontaneous $Ca^{2+}$ leak and the

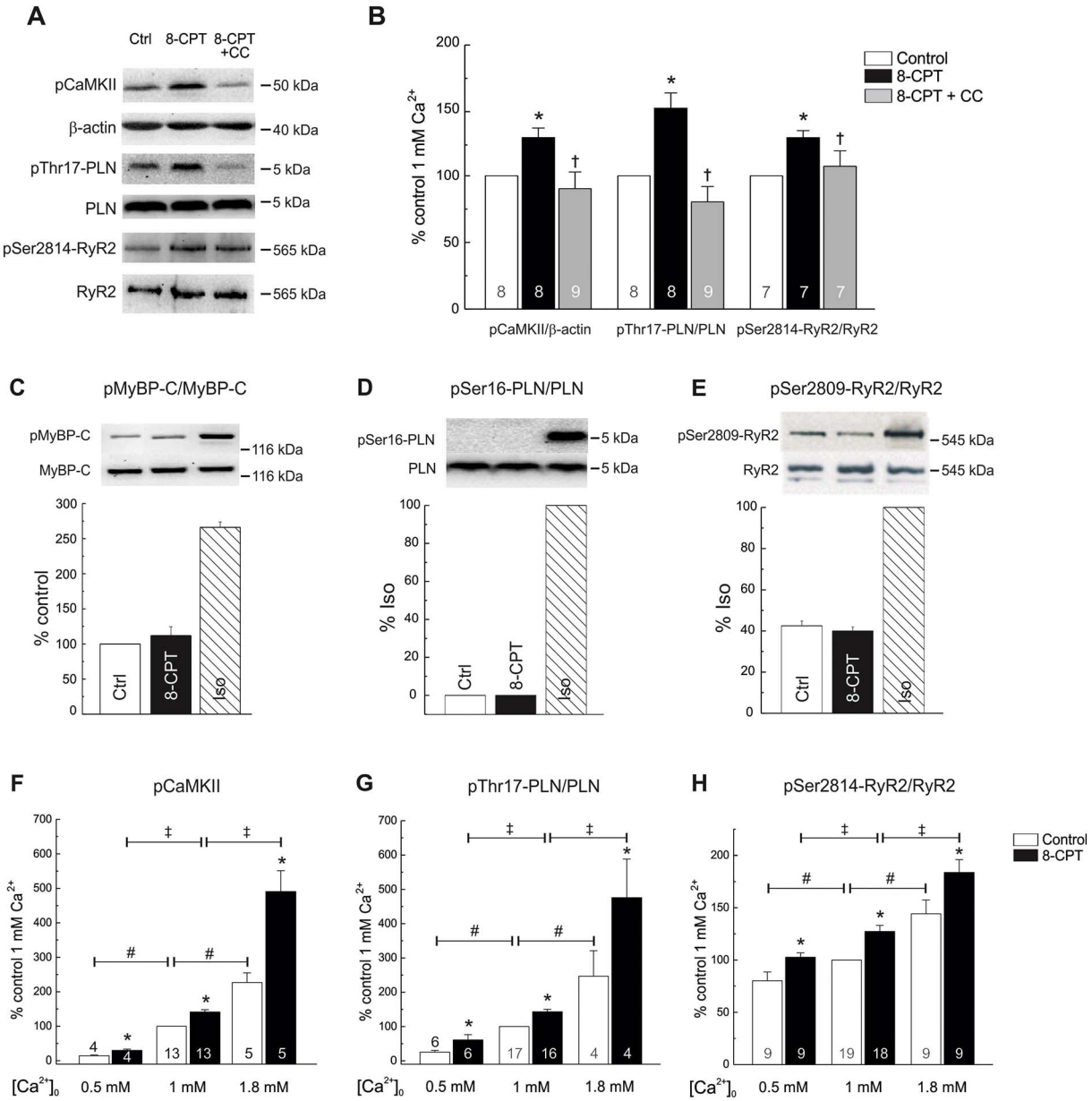


**Fig. 3.** Epac stimulation increases CaMKII-dependent phosphorylation of its substrates via PKC at different $[Ca^{2+}]_o$. Typical immunoblots (A) and overall results (B) of the autophosphorylation of CaMKII (Thr286 site) and site-specific CaMKII phosphorylation of PLN (Thr17) and RyR2 (Ser2814) in rat myocytes incubated in the absence (white bars) or presence of 10 μM 8-CPT (black bars) or in the simultaneous presence of 8-CPT and the PKC inhibitor, 0.5 μM calphostine C (CC, grey bars). * $p < 0.05$ vs. control, in the absence of drug; † $p < 0.05$ vs. 8-CPT. C, D and E, typical immunoblots and overall results of the phosphorylation of myosin binding protein C (MyBP-C, Ser282) and the site-specific PKA phosphorylation of PLN (Ser16) and RyR2 (Ser2808), respectively. Cardiac myocytes incubated with 1 μM Isoproterenol (Iso) were used as positive controls. Experiments in A-E were performed at 1 mM $[Ca^{2+}]_o$. F, G and H, CaMKII phosphorylation of the kinase, PLN and RyR2 at different $[Ca^{2+}]_o$. Numbers in bars indicate number of cells from at least 5 rats. * $p < 0.05$ vs. control at each $[Ca^{2+}]_o$. # $p < 0.05$ vs. 1 mM $[Ca^{2+}]_o$ in the absence of drug. Ctrl, Control.

consequent arrhythmogenesis at high $[Ca^{2+}]_o$, consistent with previous studies [6,10,13]. The results would also indicate that at each experimental condition the resultant effect of Epac activation is dependent on the prevailing mechanism of SR $Ca^{2+}$ handling, i.e. uptake or leak.

## 4. Discussion

The discovery of Epac proteins expanded the range of cAMP effectors and many of the mechanisms believe to be PKA-mediated proved to be Epac-dependent. At the level of the myocardium, the currently understanding of the effects of Epac activation remains controversial. Inconsistent results have been reported regarding the impact of Epac on $Ca^{2+}$ homeostasis, contractility, apoptosis, hypertrophy and fibrosis [1,2]. The present study was carried out in order to clarify some of these discrepancies. We particularly focused on the effects of Epac activation on cardiomyocyte contractility and SR $Ca^{2+}$ handling.

The results obtained reveal that: 1) The activation of Epac promotes different effects on $Ca^{2+}$ handling and contractility in cardiac myocytes, which do not depend on the species but on the experimental conditions that alter the fine-tuning of the SR $Ca^{2+}$ handling; 2) Epac stimulation regulates both the uptake and release of $Ca^{2+}$ from the SR; 3) CaMKII is an essential element in the downstream cascade of the Epac signaling, which through PLN phosphorylation determines the positive inotropic effect of Epac at low $[Ca^{2+}]_o$ and through RyR2 phosphorylation is responsible for the arrhythmogenic effects of Epac activation at high $[Ca^{2+}]_o$ and 4) PKC is involved in the Epac-induced CaMKII activation.

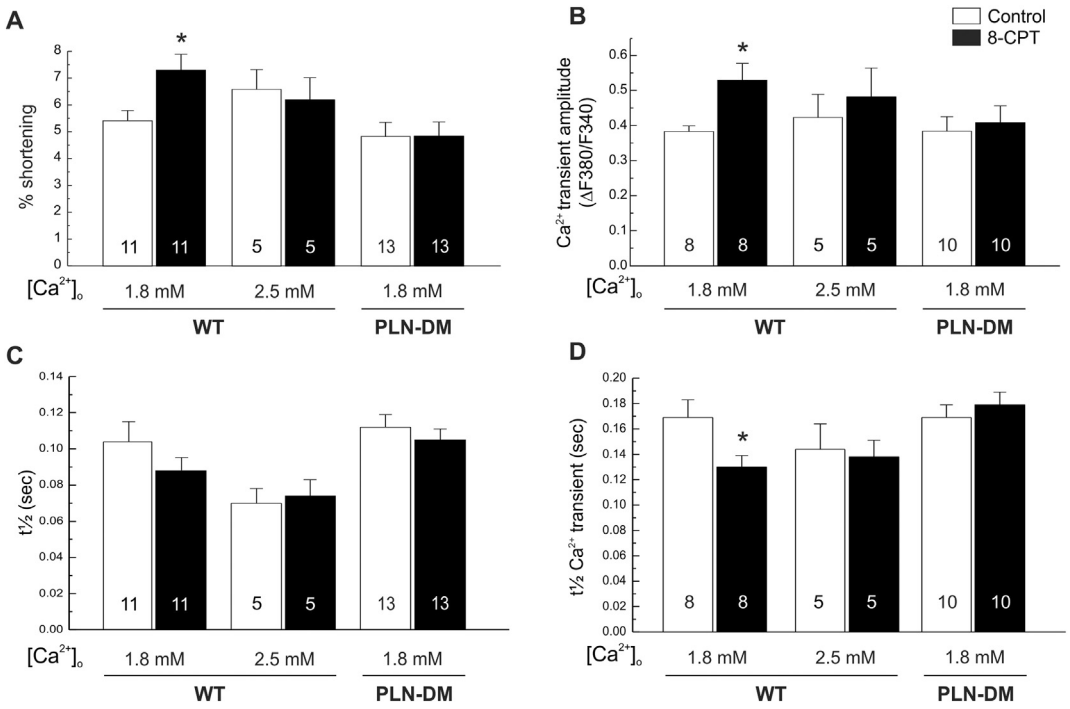


**Fig. 4.** Epac-induced positive inotropic and lusitropic effects are blunted in PLN-DM transgenic mice. A, sarcomere cell shortening; B, $Ca^{2+}$ transient amplitude; C, time to 50% relaxation (half relaxation time; t1/2) of twitches and D, time to 50% decay of calcium transient of WT and PLN-DM mouse myocytes loaded with Fura-2 AM and field-stimulated at 1 Hz, before (white bars) and during (black bars) 10 μM 8-CPT application at different $[Ca^{2+}]_o$. * $p < 0.05$ paired *t*-test vs. before 8-CPT stimulation at each $[Ca^{2+}]_o$. Numbers in bars indicate number of cells of at least 5 mice.

### 4.1. Epac in $Ca^{2+}$ handling and contractility

The present results showed that at low $[Ca^{2+}]_o$, the Epac-specific agonist, 8-CPT, produced positive inotropic and relaxant effects, both in rat and mouse myocytes. These effects were associated with an increased SR $Ca^{2+}$ load suggesting an enhancement of the SR $Ca^{2+}$ uptake through the SERCA2a/PLN complex. The increment of CaMKII-dependent PLN phosphorylation and the use of PLN-DM mice definitively confirmed that an increase in SR $Ca^{2+}$ uptake was underlying the contractile changes induced by Epac. CaMKII-dependent RyR2 phosphorylation also occurred associated to an increase in the spontaneous SR $Ca^{2+}$ release. However, this $Ca^{2+}$ leak seems to affect neither the SR $Ca^{2+}$ load nor the diastolic $Ca^{2+}$. We believe that at low $[Ca^{2+}]_o$, the stimulation of the uptake through the SERCA2a/PLN complex prevails over the leak of $Ca^{2+}$ through the RyR2. However, this mechanism has been underestimated when the effects of Epac on $Ca^{2+}$ handling and contractility was studied. A different situation occurred when Epac activation was studied at higher $[Ca^{2+}]_o$. In this environment, no contractile changes or a negative inotropic effect was detected. The Epac selective agonist produced a massive increase in SR $Ca^{2+}$ leak -visualized by the presence of $Ca^{2+}$ waves-, the increment in diastolic $Ca^{2+}$ and the appearance of arrhythmias. These events occurred at reduced SR $Ca^{2+}$ content, indicating a hyperactive RyR2. As in low $[Ca^{2+}]_o$, 8-CPT treatment increased both CaMKII-dependent RyR2 and PLN phosphorylation, however under this condition, RyR2 phosphorylation-mediated $Ca^{2+}$ leak seemed to prevail over the PLN phosphorylation-mediated increase in SR $Ca^{2+}$ uptake, leading to a loss in SR $Ca^{2+}$ content. The lack of arrhythmic events in 8-CPT treated myocytes from RyR2-S2814A mice confirmed that CaMKII-dependent RyR2 phosphorylation was responsible for the hyperactivity of the channel in this situation. Taken together, the present results indicate that Epac activation exacerbates the predominant mechanism of SR $Ca^{2+}$ handling at each experimental condition (net $Ca^{2+}$ accumulation vs. net $Ca^{2+}$ release).

Taking this into consideration, the diverse effects of 8-CPT on intracellular $Ca^{2+}$ homeostasis and contractility found by different authors could be comprehended. For instance, Oestreich et al. [5] using mouse myocytes at 2 mM $[Ca^{2+}]_o$, close to our 1.8 mM $[Ca^{2+}]_o$ (low $[Ca^{2+}]_o$ for the mice) reported an increased $Ca^{2+}$ transient amplitude in response to Epac stimulation whereas Pereira et al. [6,10] and Cazorla et al. [9] working with rat myocytes at 1.8 mM $[Ca^{2+}]_o$, situation of high $[Ca^{2+}]_o$ in this species, found a reduced $Ca^{2+}$ transient amplitude. In the acute stimulation of Epac, the participation of other non−SR proteins involved in the regulation of intracellular $Ca^{2+}$ homeostasis such as the L-type $Ca^{2+}$ channel [6] or the NCX (6, 10 and the present work) seemed unlikely.

Recently, the effect of Epac activation under different $[Ca^{2+}]_o$ was studied in a rat multicellular ventricular preparation [19]. Similarly to our results, Kaur et al. described a positive inotropic effect of Epac activation at low $[Ca^{2+}]_o$ (0.5 mM) and a lack of effect when $[Ca^{2+}]_o$ increased (1.5 mM). The authors attributed to a CaMKII-mediated enhancement in myofilament $Ca^{2+}$ sensitivity the increase in contractility observed at 0.5 mM $[Ca^{2+}]_o$. A previous evidence presented by Cazorla et al. [9] also supported an Epac-induced sensitization of myofilament to $Ca^{2+}$ in rat permeabilized cardiomyocytes. This effect was associated to a PKC and CaMKII-dependent increase in MyBPC and troponin-I phosphorylations. Notably, our present results are at odds with these previous studies. We were not able to detect changes in either apparent myofilament $Ca^{2+}$ sensitivity or phosphorylation of MyBPC in response to 8-CPT. We cannot yet explain this negative result and certainly more experiments would be necessary to elucidate the role of Epac as regulator of myofilament function, which has been scarcely studied.

### 4.2. Arrhythmogenic effects of Epac

The arrhythmogenic effects of acute Epac stimulation were first reported by Hothi et al. [11]. The fact that 8-CPT promoted ventricular arrhythmogenesis without changes in action potential duration, transmural repolarization gradient or ventricular refractoriness in isolated perfused mouse hearts and that the Epac agonist induced spontaneous

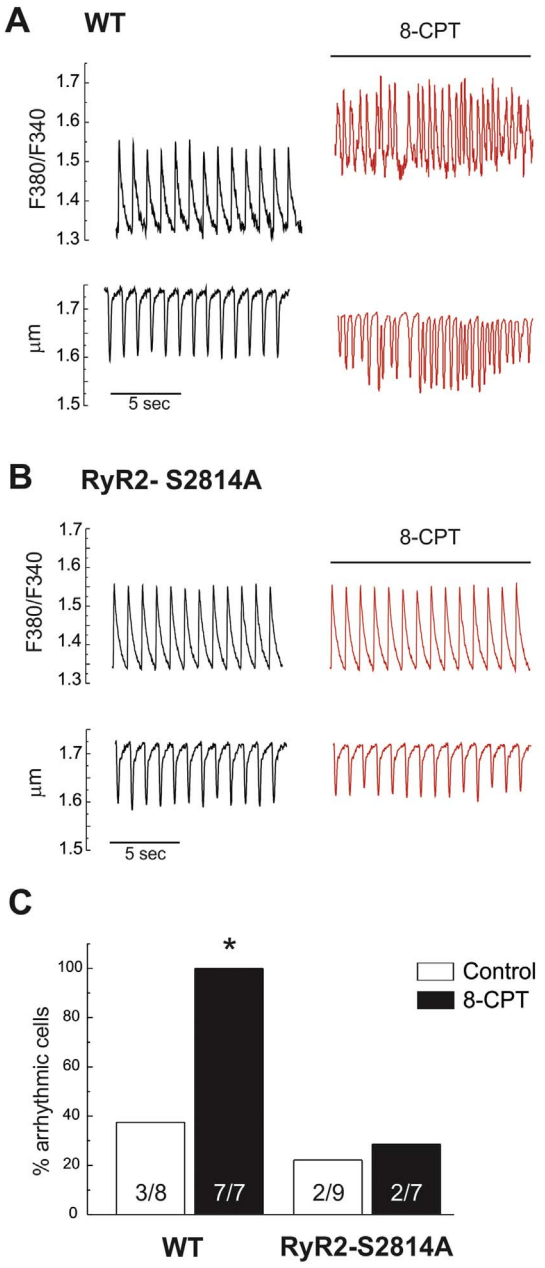


**Fig. 5.** Epac-induced arrhythmogenesis is prevented in RyR2S2814A transgenic mice. Representative recordings of $Ca^{2+}$ transient and sarcomere cell shortening of myocytes from WT mice (A) and RyR2-S2814A mice (B) before and after 5 min of application of 10 µM 8-CPT at 2.5 mM $[Ca^{2+}]_o$. C, percentage of myocytes in each group that experienced arrhythmic episodes. Numbers in bars indicate number of cells of at least 5 mice per group. * $p < 0.05$ vs. control by Fisher's exact test for each mice.

$Ca^{2+}$ transients and $Ca^{2+}$ waves in isolated myocytes, led the authors to propose an aberrant SR $Ca^{2+}$ release as the arrhythmogenic mechanism for Epac activation. This hypothesis was later supported by the finding of 8-CPT-induced enhancement of $Ca^{2+}$ spark frequency and increase in CaMKII-dependent RyR2 phosphorylation, a posttranslational modification known to make the channel leaky [6–8,10,13]. In this study, we were able to definitively establish that CaMKII-dependent RyR2 phosphorylation is an underlying mechanism of Epac-induced arrhythmogenesis by showing that 8-CPT failed to promote spontaneous $Ca^{2+}$ transients and contractions in stimulated RyR2-S2814A mice myocytes. However, we still cannot discard the participation of other potentially arrhythmogenic targets also reported to be modulated by Epac [12,20–23]. Although in some of these studies, the effects of 8-CPT on L-type $Ca^{2+}$ channel, slowed delayed-rectifier $K^+$ current and transient receptor potential canonical (TRPC) channels were observed after sustained activation of Epac [12,21,23], in others, the effects were detected after acute stimulation of the cAMP effector [20,22].

### 4.3. Epac-induced pathways of CaMKII stimulation

Our results showed that Epac activation increased CaMKII autophosphorylation and enhanced CaMKII phosphorylation of RyR2 and PLN in a PKC-dependent manner. These results are in line with those reported by Ostreicht et al. [9] and suggest that PKC and CaMKII share a common pathway. However, the precise interaction between the two kinases is still uncertain. One possibility is that PKC can directly activate CaMKII by phosphorylating the autophosphorylation site, as described in vitro [24]. An alternative mechanism is a PKC indirect activation of CaMKII. In rat hippocampus [25,26], PKC was shown to phosphorylate proteins that lead to: 1) increased intracellular $Ca^{2+}$, 2) the release of bound calmodulin, making it available for CaMKII and 3) the redistribution of CaMKII, modifying its proximity to target proteins. Furthermore, a new signaling pathway involving PI3K/NOS has recently been reported to mediate Epac-induced CaMKII activation [8]. The evidence presented up to now, does not allow us to elucidate whether different CaMKII activation pathways during Epac stimulation work in parallel or constitute intermediaries of a single pathway.

## 5. Conclusion

Our study demonstrates that the effects of Epac activation are strongly conditioned by the steady-state $Ca^{2+}$ levels at which the myocyte is functioning. As intracellular $Ca^{2+}$ increases, the activity of CaMKII enhances and the phosphorylation of its SR substrates acquires different relevance in the control of SR $Ca^{2+}$ handling. The $Ca^{2+}$ uptake stimulated by the CaMKII-dependent PLN phosphorylation is gradually overcome by the $Ca^{2+}$ leak facilitated by the CaMKII-dependent RyR2 phosphorylation. In this scenario, stimulation of Epac through the Epac/PKC/CaMKII exacerbates the prevailing mechanism. Therefore, under different physiological or pathological situations, this cAMP-induced signaling pathway may produce beneficial (increased contractility) or detrimental (impaired contractility and triggered arrhythmias) effects depending on the myocyte intracellular $Ca^{2+}$ availability, dynamically regulated by the balance between SR $Ca^{2+}$ uptake and leak.

## Conflict of interest

None declared.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

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