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

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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

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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