Journal of Molecular and Cellular Cardiology 52 (2012) 283–291

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Epac enhances excitation–transcription coupling in cardiac myocytes

Laetitia Pereira [a,b,1], Gema Ruiz-Hurtado [a,c,1], Eric Morel [c], Anne-Coline Laurent [c,d], Mélanie Métrich [c,d], Alejandro Domínguez-Rodríguez [c], Sandra Lauton-Santos [a], Alexandre Lucas [c,d], Jean-Pierre Benitah [a,c], Donald M. Bers [b], Frank Lezoualc'h [c,d], Ana M. Gómez [a,c,*]

[a] Inserm, U637, Université de Montpellier 1, Université de Montpellier 2, France
[b] Department of Pharmacology, University of California Davis, Davis, USA
[c] Inserm, U769, Univ. Paris-Sud, IFR141, Labex Lermit, Châtenay-Malabry, France
[d] Inserm, UMR 1048, I2MC, Univ. Paul Sabatier, Toulouse, France

ARTICLE INFO

Article history:
Received 28 September 2011
Accepted 19 October 2011
Available online 29 October 2011

Keywords:
Calcium signaling
Excitation–contraction coupling
Epac
Excitation–transcription coupling
Ventricular myocyte

ABSTRACT

Epac is a guanine nucleotide exchange protein that is directly activated by cAMP, but whose cardiac cellular functions remain unclear. It is important to understand cardiac Epac signaling, because it is activated in parallel to classical cAMP-dependent signaling via protein kinase A. In addition to activating contraction, Ca^{2+} is a key cardiac transcription regulator (excitation–transcription coupling). It is unknown how myocyte Ca^{2+} signals are decoded in cardiac myocytes to control nuclear transcription. We examine Epac actions on cytosolic ($[Ca^{2+}]_i$) and intranuclear ($[Ca^{2+}]_n$) Ca^{2+} homeostasis, focusing on whether Epac alters $[Ca^{2+}]_n$ and activates a prohypertrophic program in cardiomyocytes. Adult rat cardiomyocytes, loaded with fluo-3 were viewed by confocal microscopy during electrical field stimulation at 1 Hz. Acute Epac activation by 8-pCPT increased Ca^{2+} sparks and diastolic $[Ca^{2+}]_i$, but decreased systolic $[Ca^{2+}]_i$. The effects on diastolic $[Ca^{2+}]_i$ and Ca^{2+} spark frequency were dependent on phospholipase C (PLC), inositol 1,4,5 triphosphate receptor (IP_3R) and CaMKII activation. Interestingly, Epac preferentially increased $[Ca^{2+}]_n$ during both diastole and systole, correlating with the perinuclear expression pattern of Epac. Moreover, Epac activation induced histone deacetylase 5 (HDAC5) nuclear export, with consequent activation of the prohypertrophic transcription factor MEF2. These data provide the first evidence that the cAMP-binding protein Epac modulates cardiac nuclear Ca^{2+} signaling by increasing $[Ca^{2+}]_n$ through PLC, IP_3R and CaMKII activation, and initiates a prohypertrophic program via HDAC5 nuclear export and subsequent activation of the transcription factor MEF2.

1. Introduction

Ca^{2+} is a key element in cardiac excitation–contraction (EC) coupling. In each heartbeat, membrane depolarization during an action potential activates L-type Ca^{2+} channels located in the sarcolemma. Ca^{2+} entry through these channels activates intracellular Ca^{2+} release channels, named ryanodine receptors (RyRs), which are located in the membrane of the sarcoplasmic reticulum (SR). RyRs amplify the initial Ca^{2+} signal via Ca^{2+}-induced Ca^{2+} release (CICR), providing enough Ca^{2+} to activate contractile myofibrils. Relaxation then occurs when intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) returns to diastolic values, due mainly to Ca^{2+} pumped back into the SR by the Ca^{2+}-ATPase (SERCA) and extrusion from the cell via Na^+/Ca^{2+} exchange (NCX) [1]. While Ca^{2+} in EC coupling is physiologically and pathophysiologically relevant, new roles for cardiac myocyte Ca^{2+} are being elucidated. For instance, prohypertrophic signaling seems to be activated by perinuclear activation of Ca^{2+}/calmodulin dependent protein kinase II (CaMKII) promoted by local elevation of nuclear $[Ca^{2+}]$ ($[Ca^{2+}]_n$) [2]. By analogy to EC coupling, this process has been named

Abbreviations: $[Ca^{2+}]$, calcium concentration; $[Ca^{2+}]_i$, intracytoplasmic calcium concentration; $[Ca^{2+}]_n$, intranuclear calcium concentration; 2-APB, 2-aminoethoxydiphenyl borate; 8-pCPT, 8-(4-chlorophenylthio)-2-*O*-methyladenosine-3,5_-cyclic monophosphate; BP, band Pass; BSA, bovine serum albumin; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CICR, Ca^{2+}-induced Ca^{2+} release; DAPI, 4′,6′-diamidino-2′-phenylindoladihydrochloride; EC, excitation–contraction; ET, excitation–transcription; ET-1, endothelin-1; GEF, guanylyl exchange protein; GFP, green fluorescence protein.; HDAC5, histone deacetylase 5; IP_3, inositol 1,4,5 triphosphate; IP_3R, inositol 1,4,5 triphosphate receptor; MEF2, myocyte enhancer factor 2; MEF2-Luc, 3xMEF2-luciferase reporter gene; n.a., numeric aperture; NCX, Na^+/Ca^{2+} exchange; o.i., oil inmersion; PBS, phosphate buffered saline; PKA, protein kinase A; PKC, Protein Kinase C; PLC, phospholipase C; RT, room temperature; RyR, ryanodine receptor; SDS, sodium dodecyl sulphate; SERCA, Sarco/endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum; w.i., water immersion.

* Corresponding author at: Inserm U769, Faculté de Pharmacie, Tour D4, Université de Paris Sud, 92296 Châtenay-Malabry, France. Tel.: +33 146 83 57 18; fax: +33 1 46 83 54 75.
E-mail address: ana-maria.gomez@inserm.fr (A.M. Gómez).
[1] Equal contributors.

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doi:10.1016/j.yjmcc.2011.10.016

excitation–transcription (ET) coupling. However, it is still not fully understood how $[Ca^{2+}]_n$ variations may be dissociated from bulk $[Ca^{2+}]_i$ oscillations during contraction–relaxation cycles. We recently showed that Epac (exchange protein directly activated by cAMP) could activate SR Ca^{2+} release in ventricular myocytes, via a CaMKII-dependent phosphorylation of the RyR [3], and we hypothesized that Epac-dependent Ca^{2+} signaling may also be implicated in cardiac hypertrophy [4,5].

Epac induces cardiomyocyte hypertrophy, both in cultured neonatal ventricular myocytes [4] and in adult myocytes [5]. Epac is a guanylyl exchange protein (GEF) [6,7] widely distributed in the organism, including the heart, whose functional roles are just beginning to be defined [8,9]. The hypertrophic effects of Epac are independent of the classical effector of cAMP, protein kinase A (PKA), but rather involve the CaMKII [5]. In addition, Epac expression is increased in experimental animal models of cardiac hypertrophy [10] and contributes to the hypertrophic effect of β-adrenergic receptor [5]. CaMKII is also known to activate nuclear export of class II histone deacetylases (e.g. HDAC4 and 5) [2,11], an effect which derepresses myocyte enhancer factor 2 (MEF2) driven transcription, and contributes to hypertrophic remodeling.

Here we dissected the signaling pathway linking Epac activation to Ca^{2+} mobilization, paying a special attention to $[Ca^{2+}]_n$, and activation of HDAC5 nuclear export. By simultaneously analyzing cytoplasmic and $[Ca^{2+}]_n$ during selective Epac activation with 8-pCPT-2′-O-Me-cAMP (8-pCPT), we found that Epac preferentially and differently increases $[Ca^{2+}]_n$ (more than bulk $[Ca^{2+}]_i$). This action involves phospholipase C (PLC)/Inositol 1,4,5-trisphosphate (IP_3) signaling and CaMKII activation. In addition, we found that Epac induced HDAC5 export via activation of IP_3R and CaMKII, and results in activation of the hypertrophic transcription factor MEF2.

2. Methods

All experiments were carried out according to the ethical principles laid down by the French (Ministry of Agriculture) and European Union Council Directives for the care of laboratory animals.

2.1. Cell isolation and Ca^{2+} imaging

Ventricular cardiomyocytes from Wistar rats (250–300 g) were isolated using a standard enzymatic digestion as previously described [12]. Only rod-shaped cells, quiescent when unstimulated and excitable were used for the Ca^{2+} experiments.

$[Ca^{2+}]_i$ transients and Ca^{2+} sparks were recorded in intact myocytes loaded with fluorescent Ca^{2+} dye (Fluo-3 AM) [13] and under control Tyrode perfusion (in mmol/L): 140 NaCl, 4 KCl, 1.1 $MgCl_2$, 10 HEPES, 10 glucose, 1.8 $CaCl_2$; pH = 7.4, with NaOH. To record $[Ca^{2+}]_i$ transients, cells were excited at 1 Hz by field stimulation using two parallel Pt electrodes. Spontaneous Ca^{2+} sparks were obtained in quiescent cells after $[Ca^{2+}]_i$ transients recordings.

To record Ca^{2+} fluorescence in the Ca^{2+} stores (SR and nuclear envelope) cells were loaded with the low-affinity Ca^{2+} dye Mag-Fluo-4 AM (6 μmol/L) at 37 °C for 60 min allowing the compartmentalization of the dye for intra store $[Ca^{2+}]$ measurements.

Images were obtained with confocal microscopy (Meta Zeiss LSM 510, objective w.i. 63×, n.a. 1.2) by scanning the cell with an Argon laser every 1.54 ms; fluorescence was excited at 488 nm and emissions were collected at >505 nm. Image analyses were performed by homemade routines using IDL software (Research System Inc.). Images were corrected for the background fluorescence. Ca^{2+} sparks were detected using an automated detection system and a criterion that limited the detection of false events while detecting most Ca^{2+} sparks [14].

2.2. Cardiac myocytes transfection

For HDAC5 transfection, isolated adult rat cardiomyocytes were seeded on laminin-coated glass coverslips and cultured in a PC-1™ complete serum-free medium (Lonza) completed with penicillin (5%) and streptomycin (5%). Nonadherent cells were removed after 30–45 min. The myocytes were then exposed overnight to recombinant replication-deficient adenovirus expressing HDAC5-GFP (100 multiplicities of infection). HDAC5-GFP signal was studied in control condition, under endothelin-1 (ET-1) (100 nmol/L) and upon 8-pCPT (10 μmol/L) exposure. HDAC5-GFP signals were assessed every 10 min for 60 min by confocal microscopy (Zeiss LSM 5 Live, objective o.i. 40×). Image-J software was used for image analysis.

For MEF2 activity, neonatal rat ventricular myocytes were isolated [15]. Transient transfection experiments were performed with Lipofectamine 2000 (Invitrogen Life Technologies, France) in optimum medium in the presence of 1 μg of the plasmid constructs according to the manufacturers' instructions. The Epac1 plasmid construct and 3xMEF2-luciferase reporter gene (MEF2-Luc) constructs were provided by Dr. J. Bos (University of Utrecht, The Netherlands) and Dr K.C. Wollert (Hanover Medical School, Germany), respectively.

2.3. Immunolabeling

Freshly isolated adult cadiomyocytes were fixed in 4% paraformaldehyde for 10 min and washed two times in phosphate buffered saline (PBS) solution for 15 min. Myocytes were incubated in a solution of 1% bovine serum albumin (BSA) and 5 mg/mL saponin in PBS for 1 h at room temperature (RT). Myocytes were incubated with anti-Epac antibody (1:100, Santa Cruz Biotechnology, Inc) in PBS solution with 1% BSA overnight at 4 °C. Then myocytes were washed 4 times every 30 min with 1% BSA in PBS solution and incubated with the secondary antibody goat anti-rabbit Alexa 488 in the same solution for 2 h at RT. 4′,6′-diamidino-2′-phenylindoladihydrochloride (DAPI) was used as nuclear counterstain and was added to the secondary antibody solution in a final concentration of 1 μg/mL. After a serial of washes (first every 30 min for 2 times with 1% BSA in PBS solution, and later 2 times 15 min each in PBS), samples were mounted on glass slides in Slow Fade Lite (Invitrogen Corp.). For negative control we used the same protocol omitting the primary antibody in the solution. Images were recorded using confocal microscopy (Meta Zeiss LSM 510, objective w.i. 63×). Alexa fluorescence was excited by the 488 nm line of an Argon laser and emission collected through a BP filter (505–530 nm). DAPI fluorescence was excited by the Hg lamp (BP 365/12) and emission collected through a BP filter (480–520 nm).

2.4. Immunoblot

Adult cardiac myocytes treated or not with 8-pCPT were lysed in a buffer containing 50 mmol/L Tris pH 7.5, 500 mmol/L NaCl, 20 mmol/L MgCl2, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulphate (SDS), and protease inhibitors (1 mmol/L Phenylmethylsulphonylfluoride, 10 μg/L aprotinine, 10 μg/L leupeptin). After centrifugation for 20 min at 15,000 g, proteins were denaturated in Laemmli's buffer (10 min, 37 °C), separated on SDS-polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene difluoride membrane (Hybond-P; GE Healthcare). Membranes were hybridized overnight at 4 °C with the primary antibody against phosphorylated RyR at Ser2815 site (gift from Dr. A.R. Marks [16]) or RyR (Affinity BioReagents).

2.5. Chemical products

8-pCPT was purchased from Sigma and used at a final concentration of 10 μmol/L. CaMKII inhibitor KN93 (1 μmol/L) was purchased from Calbiochem and PLC inhibitor U73122 (2 μmol/L) from Tocris. IP_3R