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Epac enhances excitation-transcription coupling in cardiac myocytes

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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 ($[\text{Ca}^{2+}]_i$) and intranuclear ($[\text{Ca}^{2+}]_n$) Ca^{2+} homeostasis, focusing on whether Epac alters $[\text{Ca}^{2+}]_n$ 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 $[\text{Ca}^{2+}]_i$, but decreased systolic $[\text{Ca}^{2+}]_i$. The effects on diastolic $[\text{Ca}^{2+}]_i$ and Ca^{2+} spark frequency were dependent on phospholipase C (PLC), inositol 1,3,5 triphosphate receptor (IP_3R) and CaMKII activation. Interestingly, Epac preferentially increased $[\text{Ca}^{2+}]_n$ 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 $[\text{Ca}^{2+}]_n$ through PLC, IP_3R and CaMKII activation, and initiates a prohypertrophic program via HDAC5 nuclear export and subsequent activation of the transcription factor MEF2.

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Disclosures

None

Keywords

Calcium signaling; excitation-contraction coupling; Epac; excitation-transcription coupling; ventricular myocyte

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 ($[\text{Ca}^{2+}]_i$) 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 $\text{Na}^+/\text{Ca}^{2+}$ 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 CaMKII promoted by local elevation of nuclear $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_n$).[2] By analogy to EC coupling, this process has been named excitation-transcription (ET) coupling. However, it is still not fully understood how $[\text{Ca}^{2+}]_n$ variations may be dissociated from bulk $[\text{Ca}^{2+}]_i$ 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 Ca^{2+} /calmodulin-dependent protein kinase II (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 $[\text{Ca}^{2+}]_n$, and activation of HDAC5 nuclear export. By simultaneously analyzing cytoplasmic and $[\text{Ca}^{2+}]_n$ during selective Epac activation with 8-pCPT-2'-O-Me-cAMP (8-pCPT), we found that Epac preferentially and differently increases $[\text{Ca}^{2+}]_n$ (more than bulk $[\text{Ca}^{2+}]_i$). 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_3 R 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.

$[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ 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 μM /L) at 37°C for 60 min allowing the compartmentalization of the dye for intra store $[\text{Ca}^{2+}]_i$ measurements.

Images were obtained with confocal microscopy (Meta Zeiss LSM 510, objective w.i. 63x, 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-1TM 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 μM /L) exposure. HDAC5-GFP signals were assessed every 10 minutes for 60 minutes by confocal microscopy (Zeiss LSM 5 Live, objective o.i. 40x). 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 cardiomyocytes 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 1h 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 hours 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. 63x). Alexa fluorescence was excited by the 488nm 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 aprotinin, 10 µg/L leupeptin). After centrifugation for 20 min at 15000 g, proteins were denatured 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. IP₃R antagonist 2-aminoethoxydiphenyl borate (2-APB) (2 µmol/L) and PKC inhibitor chelerythrine (2 µM) were purchased from Sigma.

2.6 Statistical analysis

Data are presented as means±SEM. Statistical significance was evaluated by analysis of Students *t*-test paired or unpaired whenever appropriate. Differences with values of *p*<0.05 were considered statistically significant.

3. Results

3.1 Epac effect on intracellular Ca²⁺

To simultaneously analyze Epac effects on cytosolic ([Ca²⁺]_i) and [Ca²⁺]_n, fluo-3 loaded isolated adult ventricular myocytes field-stimulated at 1 Hz were subjected to line scans including the nucleus, and normalized to the diastolic fluorescence in each compartment (F₀) [17]. We are not comparing [Ca²⁺]_n vs. [Ca²⁺]_i in absolute terms, but separately assessing how Epac alters [Ca²⁺]_n and [Ca²⁺]_i. Figure 1A shows representative line scan images from a cardiomyocyte paced (1 Hz) before (left) and during (right) 8-pCPT exposure to selectively activate Epac.[18] Figure 1B shows that [Ca²⁺]_n transients (red) are delayed with respect to [Ca²⁺]_i transients (green) and have much slower decay (Supplemental Fig. S1). Moreover, the amplitudes (ΔF/F₀) of nuclear [Ca²⁺]_n transients are smaller than those in the cytosol (Fig. 1C).

Application of 8-pCPT increased both diastolic [Ca²⁺]_n and [Ca²⁺]_i, with a larger increase in [Ca²⁺]_n (as %; Fig. 1D). Exposure to 8-pCPT caused reduction of peak systolic [Ca²⁺]_i and [Ca²⁺]_i transient amplitude (Fig. 1C&D), but enhanced peak systolic [Ca²⁺]_n (Fig. 1D). In the nucleus, this combination of effects (increase of both, diastolic and systolic [Ca²⁺]_n) resulted in no significant change in the amplitude of the [Ca²⁺]_n transient (Fig. 1C). The overall elevation of [Ca²⁺]_n in response to 8-pCPT (both diastolic and peak) (Fig. 1D) suggests a preferential effect of 8-pCPT on nuclear vs. cytoplasmic [Ca²⁺], which may

influence nuclear Ca^{2+} -dependent signaling. Similar data were observed with 10 fold lower 8-pCPT concentration (supplementary Fig. S2)

3.2 Mechanism of Epac effect on Diastolic and Systolic $[\text{Ca}^{2+}]_i$

In assessing the mechanism of Epac induced alterations in Ca^{2+} signaling, we monitored both diastolic and systolic $[\text{Ca}^{2+}]_i$ simultaneously at 1Hz stimulation (Fig. 2). Although fluo-3 is non-ratiometric, data were collected in the same cell before and during 8-pCPT application, allowing assessment of Epac effects on $[\text{Ca}^{2+}]_i$ (i.e. normalization vs. the same cell before Epac activation). A time control without Epac activation shows that the $[\text{Ca}^{2+}]_i$ signals were stable during the 5-7 min recording period (Fig. 2A-B, light grey bars). Epac can activate phospholipase C (PLC) in mouse cardiomyocytes[19], and that could mediate these effects via consequent PKC and IP_3 activation. Moreover, IP_3R are concentrated at the nuclear envelope[20] and associated with CaMKII in ventricular myocytes.[21] These pathways were tested.

We observed that Epac activation significantly increased diastolic $[\text{Ca}^{2+}]_i$ (Fig. 2A), and this effect was completely prevented by inhibition of either phospholipase C (PLC; by 2 μM U73122), the IP_3R (by 2 μM 2-aminoethoxydiphenyl borate, 2-APB), or CaMKII (by 1 μM KN-93). Inhibition of protein kinase C (PKC by 2 $\mu\text{mol/L}$ chelerythrine[22]) partially, but incompletely inhibited the Epac-induced elevation of diastolic $[\text{Ca}^{2+}]_i$ (Fig. 2A). These results suggest that Epac-induced elevation of diastolic $[\text{Ca}^{2+}]_i$ may be attributable to activation of PLC, IP_3R and CaMKII (with possible involvement of PKC). Similar results were obtained in quiescent cells: 8-pCPT treatment induced an increase on resting Ca^{2+} fluorescence by 23.25% ($p < 0.05$, $n = 14$) that was inhibited in the presence of PLC blocker (5.63% increase, N.S., $n = 9$), or in the presence of IP_3R blocker (1.23% increase, N.S., $n = 14$), and CaMKII blocker (0.72% increase, N.S., $n = 13$). This increase in resting Ca^{2+} was maintained in the presence of the PKC blocker (29.8%, $p < 0.05$, $n = 9$). The increase in diastolic $[\text{Ca}^{2+}]_i$ is likely not due to an increase in Ca^{2+} entry. The main Ca^{2+} entry pathway in cardiac myocytes are the L-type Ca^{2+} channels and we [3] and others [23] previously showed that 8-pCPT did not modified the L type Ca^{2+} current. Although not prominent in these cells, store-operated Ca^{2+} entry (SOCE) may exist in cardiac myocytes and could contribute to Epac effects. This hypothesis was tested but, under our conditions, 8-pCPT failed to induce Ca^{2+} entry through SOCE (data not shown).

Epac has the opposite effect on systolic $[\text{Ca}^{2+}]_i$, showing a reduction within 2-3 min of 8-pCPT application (Fig. 2B). Both PLC and CaMKII inhibition prevented the 8-pCPT-induced decrease in systolic $[\text{Ca}^{2+}]_i$ (Fig. 2B), while PKC and IP_3R inhibition did not prevent the Epac-dependent reduction of systolic $[\text{Ca}^{2+}]_i$. Taken together, these data suggest that Epac-induced activation of PLC and CaMKII seem to be important in both the diastolic and systolic $[\text{Ca}^{2+}]_i$ modulation, while the IP_3R are more centrally involved in the diastolic $[\text{Ca}^{2+}]_i$ increase.

3.3 Ca^{2+} sparks

The 8-pCPT-induced increase of diastolic $[\text{Ca}^{2+}]_i$ may be due to increased SR Ca^{2+} leak through RyRs, but could also be secondary to slowed $[\text{Ca}^{2+}]_i$ decline[3] (Supplemental Fig. S1) during the $[\text{Ca}^{2+}]_i$ transient or alterations in trans-sarcolemmal Ca^{2+} fluxes. Epac activation did not alter the rate of Ca^{2+} extrusion via $\text{Na}^+/\text{Ca}^{2+}$ exchange or Ca^{2+} influx via Ca^{2+} current, but can increase diastolic Ca^{2+} sparks in a CaMKII-dependent manner.[3] Figure 3A shows line scan images of Ca^{2+} sparks recorded in control conditions after drug preincubation (top) and in the same cells during application of 10 $\mu\text{mol/L}$ 8-pCPT (bottom). The expected increase in Ca^{2+} spark frequency[3] was completely prevented by PLC inhibition (Fig. 3B), but not by PKC inhibition, consistent with the effects on diastolic

$[Ca^{2+}]_i$ in Figure 2. In the continuous presence of 2-APB, Epac activation was unable to significantly increase Ca^{2+} spark frequency. These results also rule out a possible direct effect of 8-pCPT on RyR2. Thus Ca^{2+} spark frequency data were consistent with those of diastolic $[Ca^{2+}]_i$. Similar to $[Ca^{2+}]_i$ transient amplitude, Ca^{2+} spark amplitudes were decreased by 8-pCPT ([3], Fig. 3C), and its effect was blocked by either PLC inhibition (Fig. 3C) or CaMKII inhibition [3], thus being consistent with systolic $[Ca^{2+}]_i$.

Taken together, these data show that PLC, CaMKII and possibly IP_3/IP_3R , but not PKC signaling are involved in the Epac-induced increase of Ca^{2+} spark occurrence. This is similar to the conclusions taken from Epac effects on diastolic $[Ca^{2+}]_i$. A working hypothesis could be that Epac activates PLC and IP_3 production, which causes release of Ca^{2+} via IP_3R and consequent activation of local CaMKII to induce RyR phosphorylation thereby increasing Ca^{2+} spark frequency. In favor of this hypothesis, 8-pCPT induced RyR phosphorylation at the CaMKII site (Ser2815) which was blocked by the PLC inhibitor, U73122 (Supplemental Fig. S3).

3.4 Nuclear Epac and $[Ca^{2+}]_n$

To further analyze the effects of Epac activation on $[Ca^{2+}]_n$ during continuous field stimulation at 1Hz, we measured diastolic, systolic and time-averaged $[Ca^{2+}]_n$ during 8-pCPT exposure (Fig. 4A). The Epac-dependent increase in $[Ca^{2+}]_n$ was prevented by the IP_3R blocker, 2APB, and by the CaMKII inhibitor, KN93.

As shown in Figure 1, Epac effects $[Ca^{2+}]_n$ more than $[Ca^{2+}]_i$, and this seems to involve IP_3R activation (see above). Moreover, 8-pCPT promoted IP_3 production (Supplementary Fig. S4). Since IP_3Rs in adult ventricular cells are concentrated in the nuclear envelope and/or perinuclear regions[20, 21, 24], we tested whether Epac localization is also perinuclear, as previously suggested.[4, 5, 25] We assessed endogenous Epac localization in freshly isolated adult ventricular cardiomyocytes by immunolabeling. Figure 4B shows fluorescence images obtained from an adult cardiac myocyte double labeled with Epac antibody (top) and DAPI to localize the nuclei (middle). Epac fluorescence is seen over the background as bright dots, which were absent in negative controls (Fig. 4C). The signal showed a preferential nuclear/perinuclear localization. The nuclear envelope may act as a Ca^{2+} reservoir and it is enriched in IP_3Rs . We monitored Ca^{2+} stored in the nuclear envelope during 8-pCPT application (Fig 4D-E). Although Mag-fluo is both a Ca^{2+} and Mg^{2+} indicator, it is currently used as low sensitivity Ca^{2+} dye to image Ca^{2+} in intracellular stores. We observed that the nuclear envelope $[Ca^{2+}]$ decreased with respect to the SR $[Ca^{2+}]$ during Epac activation, indicating proportionally higher Ca^{2+} release from the nuclear envelope than from the SR.

The Epac-induced elevation of $[Ca^{2+}]_n$ and CaMKII, and prohypertrophic effect of Epac[5] raises the question whether this IP_3 and CaMKII-dependent nuclear signaling is involved in regulating Ca^{2+} -dependent transcription factors.

3.5 Effect of Epac on transcription factors

We tested whether Epac could induce HDAC5 nuclear export in an IP_3 - and CaMKII-dependent manner. Rat myocytes were infected with an adenovirus coding for GFP-HDAC5. Figure 5A-B shows that 8-pCPT induced HDAC5 translocation out of the nucleus (seen as a reduction in nuclear to cytosolic fluorescence). The extent and time course were similar to that seen for endothelin 1 (ET-1) used as positive control (Fig. 5B). Epac also increased MEF2 transcriptional activity in primary neonatal cardiac myocytes (Fig. 5C). Finally, we found that inhibiting either CaMKII or IP_3R blocked 8-pCPT-induced HDAC5

nuclear translocation (Fig. 5D). These data show that Epac activates translocation of HDAC5 in an IP₃R and CaMKII dependent manner.

4. Discussion

The present work demonstrates a novel role for endogenous Epac in cardiac Ca²⁺ and hypertrophic signaling. Moreover, the activation of Epac by cAMP in the physiological context, likely coexists with and parallels the classical cAMP-dependent signaling via PKA, but may have very divergent downstream targets. We find that acute activation of endogenous Epac elevates [Ca²⁺]_i and Ca²⁺ sparks in a manner that depends on the activation of PLC, IP₃R and CaMKII. Epac is preferentially located near the nucleus, and acute Epac activation also drives HDAC5 nuclear export (and MEF2 activation) in a manner that is IP₃R and CaMKII-dependent, paralleling the [Ca²⁺]_i elevation. We propose a working hypothesis for diastolic [Ca²⁺] schematized in Figure 6 whereby Epac activation can activate PLC, causing DAG and IP₃ production, that the IP₃ activates Ca²⁺ release via IP₃R which result in local activation of CaMKII. This CaMKII activation may then be responsible for the phosphorylation of both RyR (enhancing Ca²⁺ sparks and unloading the SR) and HDAC5 (resulting in nuclear HDAC5 export and derepression of MEF2-dependent hypertrophic transcription). Not all of the details of these two Epac-dependent pathways are fully worked out yet (see below), but this represents a novel paradigm in cAMP-dependent Ca²⁺ signaling in cardiac myocytes that influences both EC coupling and ET coupling.

4.1 Epac activates Ca²⁺ release from the SR

We previously showed in rat cardiac myocytes that Epac enhances diastolic SR Ca²⁺ release by increasing Ca²⁺ spark frequency, and that this required CaMKII activation and RyR phosphorylation at Ser2815, without significant effects on L-type Ca²⁺ current (I_{Ca}) or Ca²⁺ removal via Na⁺/Ca²⁺ exchange.[3] Others have also seen that β-adrenergic agonists can activate RyR-mediated leak by CaMKII-dependent and PKA-independent pathways.[26] We also used this strategy to activate native Epac with isoproterenol in the absence of PKA activity. Data presented in Supplementary Fig. S5 shows that β-adrenergic stimulation with isoproterenol in the presence of a PKA inhibitor, KT5722 is able to significantly increase cytosolic and nuclear [Ca²⁺]. The Epac-induced CaMKII activation[27], RyR phosphorylation and lack of I_{Ca} modulation were also confirmed by other authors in mouse cardiomyocytes.[23] In adult rat ventricular myocytes, systolic [Ca²⁺]_i transient amplitude is reduced by 8-pCPT, but this did not translate into a negative inotropic effect [3], because of a parallel increase in myofilament Ca²⁺ sensitivity.[28] Moreover, the reduction in the Ca²⁺ transient may result from the reduced SR Ca²⁺ content (secondary to increased diastolic SR Ca²⁺ leak).

Oestreich *et al.*[19] found that Epac activation by 8-pCPT enhanced twitch [Ca²⁺]_i transients in mouse (the opposite of our result), but they did not assess SR Ca²⁺ leak, Ca²⁺ sparks or SR Ca²⁺ content. This leaves open the possibility that in their mouse experiments, the SR Ca²⁺ content may not have been decreased (perhaps diastolic Ca²⁺ leak was less), allowing higher fractional SR Ca²⁺ release and [Ca²⁺]_i transients. Despite this [Ca²⁺]_i transient amplitude discrepancy (which may have plausible explanations) a central concurrent finding of both groups is that Epac activation enhances RyR activity.

Here we found that the 8-pCPT-induced increase in both diastolic [Ca²⁺]_i and Ca²⁺ sparks depend on PLC, IP₃Rs and CaMKII. While ventricular IP₃R type 2 are concentrated at the nuclear envelope, there are some in the SR (especially in atria), where they are known to be able to facilitate the activation RyR-mediated Ca²⁺ sparks.[29, 30] The PLC- and CaMKII-dependence we observe here, agrees with results from Oestreich *et al.*[19, 23] where Epac-dependent changes in Ca²⁺ handling were also abolished in PLCε knockout mice. While

their 8-pCPT effects on Ca^{2+} handling were sensitive to PKC ϵ inhibition, our effects reflect activation of the PLC product IP_3 .

This allows a working hypothesis in which Epac-dependent PLC activation causes IP_3 -induced Ca^{2+} release that can activate CaMKII, which is known to associate with the IP_3R . CaMKII can then phosphorylate the RyR to facilitate Ca^{2+} release. Indeed, CaMKII can enhance RyR activation both at rest and during EC coupling.[31, 32] An important remaining question is how and where Epac activation causes PLC activation in adult rat cardiac myocytes.

4.2 Nuclear Ca^{2+} signaling activated by Epac

The Epac effects on Ca^{2+} signaling were more prominent on diastolic $[\text{Ca}^{2+}]_n$ than $[\text{Ca}^{2+}]_i$ (a 45 vs 28% increase with respect to control). In addition, Epac activation also increased the peak systolic $[\text{Ca}^{2+}]_n$ by 22%, whereas peak systolic $[\text{Ca}^{2+}]_i$ was reduced by 9%. The simplest interpretation of this result is that the very much higher density of IP_3R at the nuclear envelope vs. SR in ventricular myocytes allows the Epac-dependent IP_3 signal to directly raise $[\text{Ca}^{2+}]_n$ (potentially aided by CaMKII-dependent activation of sparse nuclear envelope RyRs). This may be further accentuated by the preferential perinuclear location of endogenous Epac (Fig. 4). These localization data confirm previous findings with Epac overexpression in cardiomyocytes[5], and also the finding that Epac is part of a muscle-specific A-Kinase Anchoring Protein (m-AKAP) complex localized in the nuclear envelope of neonatal cardiac myocytes.[33]

The higher diastolic $[\text{Ca}^{2+}]_n$ may also partly explain why peak systolic $[\text{Ca}^{2+}]_n$ is increased despite the reduction in peak systolic $[\text{Ca}^{2+}]_i$. That is, the bolstered baseline allows even a slightly reduced SR Ca^{2+} release signal to raise $[\text{Ca}^{2+}]_n$ to a higher level. In addition, the sensitization of nuclear IP_3R upon Epac stimulation could amplify the RyR dependent release. Additionally, in the presence of IP_3 , the $\text{IP}_3\text{R2}$ is activated by increased $[\text{Ca}^{2+}]$ [34]. This sort of enhancement of nuclear Ca^{2+} transients has been reported before during agonist stimulation that increases IP_3 production.[24]. Although the 2-APB is not a completely specific IP_3R blocker, the involvement of IP_3R is further supported by the fact that PLC is identified as an upstream element in the Epac signaling cascade (effects are blocked by U73122) and by the finding that 8-pCPT treatment increases IP_3 content.

Nuclear Ca^{2+} in the vicinity of the IP_3R has been shown to modulate transcription factors, dependent at least in part by CaMKII-dependent HDAC5 phosphorylation in ET coupling. [2] Our results here would be consistent with Epac feeding in to the same nuclear IP_3R -CaMKII pathway to activate HDAC5 nuclear export and the prohypertrophic transcription factor MEF2. Indeed, the 8-pCPT-induced HDAC5 nuclear export is prevented by either IP_3R or CaMKII inhibition (Fig. 5). This pathway may also provide a mechanistic basis for our previous findings that Epac induces cardiomyocyte hypertrophy.[4, 5] Epac has also been reported to be part of a perinuclear m-AKAP complex, which might also be an important locus for Epac induced cardiac myocyte hypertrophy.[35] Taking together, these data demonstrate that Epac has a role on ET coupling.

The proposed signaling cascade is schematized in Figure 6. Epac activation of PLC induces IP_3 production, which activates IP_3R , releasing Ca^{2+} into the nucleus and also activating RyRs in the SR. The Ca^{2+} released by IP_3Rs activates CaMKII, which can phosphorylate RyRs to increase Ca^{2+} spark occurrence, and also HDAC5 to induce its translocation out of the nucleus derepressing MEF2 transcriptional activity and thereby initiating a prohypertrophic signaling.

In summary, we have identified a role of Epac on both EC and ET coupling, and involving the activation of PLC, IP₃Rs and CaMKII with effects on RyR gating and nuclear transcription. In this way, the well-known second messenger cAMP may exert actions that are parallel to, but independent of the traditional cAMP-PKA pathway. This alternative pathway must be considered in future studies of cAMP-dependent effects on both EC and ET coupling, and cardiac pathophysiological mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 ₃ R	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 immersion
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	Ca ²⁺ -ATPase
SR	sarcoplasmic reticulum
w.i.	water immersion

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Figure 1. Epac differently modulates intranuclear and cytosolic [Ca²⁺]

A. Line scan images (including nuclei) of rat ventricular myocytes before (left) and during (right) 10 μM 8-pCPT application during field stimulation at 1 Hz. **B.** Ca²⁺ fluorescence (F) traces of the images above. Green is [Ca²⁺]_i and the red is [Ca²⁺]_n. **C.** [Ca²⁺] transient amplitudes (ΔF/F₀). Green bars are cytosol (n=14) and red bars nucleus (n=13). Lighter colors are control condition, and darker colors are with 8-pCPT. **D.** 8-pCPT effect on [Ca²⁺] as percentage of the values before the drug application in cytosol (green bars) and in the nucleus (red bars). *p<0.05, **p<0.01

Figure 2. Epac differently modulates diastolic and systolic $[Ca^{2+}]_i$ in the cytosol

A. Epac-induced increase in diastolic $[Ca^{2+}]_i$ during field stimulation at 1 Hz. Fluorescence value in each cell during 8-pCPT perfusion was normalized with the value in the same cell before 8-pCPT perfusion. White bar indicate the control in every conditions (n=59), black bar indicates 8-pCPT alone (n=13). Next to the right are time controls (same perfusion time than 8-pCPT but with control solution, n=8). Further to the right, 8-pCPT effect on diastolic $[Ca^{2+}]_i$ in the presence of various inhibitors: Light grey bar indicates PLC inhibitor (2 μ mol/L U73122, n=9), hatched bar are IP₃R blocker by 2 μ mol/L 2-APB (n=16), dark are PKC inhibitor 2 μ mol/L chelerytrine (n=14), crosshatched are CaMKII inhibitor 1 μ mol/L KN93 (n=22). **B.** The same than in A but for the peak $[Ca^{2+}]_i$ fluorescence during the twitch. Bar colors and n numbers as in A. *p<0.05, **p<0.001.

Figure 3. Epac increases Ca²⁺ sparks frequency by PLC and IP₃R activation

A. Line scan images of cardiac myocytes showing Ca²⁺ sparks in several conditions: From left to right: Control, 2 μmol/L U73122 (PLC inhibitor), 2 μmol/L chelerytrine (PKC inhibitor) and 2 μmol/L 2-APB (IP₃R inhibitor). Below each image it is shown another image from each cell, recorded in the presence of 10 μmol/L 8-pCPT (Epac activator) and in the continuous presence of each inhibitor. **B.** Bar graph showing the measured Ca²⁺ spark frequency in: control cells (white bar, n=21) and cells during 8-pCPT alone (black bar, n=14), cells in the presence of the PLC inhibitor U73122 (n=10) before (light grey bar) and during (dark grey bar) 8-pCPT application, cells in the presence of the PKC inhibitor chelerytrine (n=8) before (hatched white bar) and during 8-pCPT application (black hatched bar), and cells in the presence of the IP₃R inhibitor 2-APB (n=15) before (cross hatched bar) and during 8-pCPT application (thick crosshatched bar). *p<0.05 with respect to their own control. **C.** As in B but for the Ca²⁺ spark amplitudes (peak F/F₀). N of Ca²⁺ sparks was: 201 in control, 251 in 8-pCPT, 207 in U73122, 155 in U73122 + 8pCPT, 228 in chelerytrine, 422 in chelerytrine + 8-pCPT, 298 in 2-APB and 290 in 2-APB + 8pCPT. ***p<0.001

Figure 4. Endogenous Epac preferentially localizes at perinuclear area and its activation induces $[Ca^{2+}]_n$ increment via CaMKII and IP₃Rs pathways

A. Percentage variations in the $[Ca^{2+}]_n$ induced by 10 μ mol/L 8-pCPT (n=13) and in the presence of 1 μ mol/L KN-93 (CaMKII inhibitor; n=8) or 2 μ mol/L 2-APB (IP₃R inhibitor; n=13) during constant field stimulation at 1 Hz. Increase in basal Ca^{2+} fluorescence (during diastolic period) is represented in the white bar, the maximum Ca^{2+} fluorescence (peak) is represented by the black bar and the average Ca^{2+} fluorescence (average) is represented by the grey bar. **B.** Image of a rat ventricular cardiomyocyte immunolabeled with Epac antibody and DAPI to localize the nuclei. Epac fluorescence is shown in the top, nuclei in the middle and merged image in the bottom. **C.** Negative control: cells were treated as in A but primary antibody was omitted. **D.** Details of Mag-Fluo-4-AM loaded myocytes to differentially visualize sarcoplasmic reticulum (SR) from nuclear envelope (NE) Ca^{2+} stores at control conditions (t=0) and following 20 minutes (t=20) of perfusion with control solution (top) or 10 μ mol/L 8-pCPT (bottom). **E.** Averaged traces of nuclear and cytosolic fluorescence (F_{NE}/F_{SR}) in perfused with 10 μ mol/L 8-pCPT (red, n=8) and control (black, n=5) myocytes. The afluorescence observed was normalized to the initial F_{NE}/F_{SR} ratio for each cell. *p<0.05, **p<0.01.

Figure 5. Epac activation induces nuclear export of HDAC5 via IP₃R and CaMKII

A. Rat ventricular cardiomyocyte expressing the fusion protein HDAC5-GFP before and after 60 minutes 8-pCPT (10 μ mol/L) exposure and the corresponding 8-pCPT time-dependent HDAC5-GFP fluorescence changes (normalized to t=0 min) for both cytosol and nucleus **B.** Time dependence of HDAC5 nuclear export represented as F_{Nuc}/F_{Cyt} , normalized to the initial ratio. Cells were treated by 10 μ mol/L 8-pCPT (open triangles, n=6) or 100 nmol/L Endothelin-1 (black circles, n=4) during 60 minutes. The control group (open circles) was treated the same, except without 8-pCPT or ET-1 application (n=5). **C.** Neonatal rat ventricular myocytes transfected with either an empty vector (control) or Epac1 (Epac^{WT}) and MEF2-Luc. MEF2-luciferase activity was measured 48h later. Values represent the average of 4 independent experiments performed in triplicates and are normalized to total protein and the relative level of luciferase activity in the control transfected cells was assigned a value of 100. **D.** Average of nuclear and cytosolic fluorescence ratio (F_{Nuc}/F_{Cyt}) in presence of 8-pCPT without (light grey bar, n=10) or with KN93 (dark grey bar, n=10) or 2-APB (black bar, n=9) exposure. * p<0.05, **p<0.01.

Figure 6. Proposed scheme of Epac activated hypertrophic signaling in cardiac myocytes
 Epac leads to PLC activation and IP₃ production. Activation of IP₃R induces Ca²⁺ leak from stores, which 1) sensitizes RyRs, 2) activates CaMKII and 3) induces increase in intranuclear Ca²⁺. The increase in [Ca²⁺]_i and CaMKII activation translocates HDAC out of the nucleus releasing the repression against hypertrophy development.