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cAMP-Binding Protein Epac Induces Cardiomyocyte Hypertrophy

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Abstract—cAMP is one of the most important second messenger in the heart. The discovery of Epac as a guanine exchange factor (GEF), which is directly activated by cAMP, raises the question of the role of this protein in cardiac cells. Here we show that Epac activation leads to morphological changes and induces expression of cardiac hypertrophic markers. This process is associated with a Ca^{2+}-dependent activation of the small GTPase, Rac. In addition, we found that Epac activates a prohypertrophic signaling pathway, which involves the Ca^{2+} sensitive phosphatase, calcineurin, and its primary downstream effector, NFAT. Rac is involved in Epac-induced NFAT dependent cardiomyocyte hypertrophy. Blockade of either calcineurin or Rac activity blunts the hypertrophic response elicited by Epac indicating these signaling molecules coordinate to regulate cardiac gene expression and cellular growth. Our results thus open new insights into the signaling pathways by which cAMP may mediate its biological effects and identify Epac as a new positive regulator of cardiac growth. (Circ Res. 2005;97:0-0.)

Key Words: cAMP ■ guanine nucleotide exchange factor ■ small G protein ■ transcription factor

In the heart, cyclic adenosine 3',5'-monophosphate (cAMP) regulates many physiological processes such as contractility and relaxation. Classically, these effects are attributed to activation of the hyperpolarization-activated cyclic nucleotide-gated channels and protein kinase A (PKA) by cAMP. The recent discovery of Epac as a guanine exchange factor (GEF), which is directly activated by cAMP, raises the question of the role of this protein in cardiac cells. Here we show that Epac activation leads to morphological changes and induces expression of cardiac hypertrophic markers. This process is associated with a Ca^{2+}-dependent activation of the small GTPase, Rac. In addition, we found that Epac activates a prohypertrophic signaling pathway, which involves the Ca^{2+} sensitive phosphatase, calcineurin, and its primary downstream effector, NFAT. Rac is involved in Epac-induced NFAT dependent cardiomyocyte hypertrophy. Blockade of either calcineurin or Rac activity blunts the hypertrophic response elicited by Epac indicating these signaling molecules coordinate to regulate cardiac gene expression and cellular growth. Our results thus open new insights into the signaling pathways by which cAMP may mediate its biological effects and identify Epac as a new positive regulator of cardiac growth.

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Key Words: cAMP ■ guanine nucleotide exchange factor ■ small G protein ■ transcription factor
Here we found that Epac stimulates the activity of the small GTPase, Rac, and increases the expression of hypertrophic gene markers in primary cardiac myocytes. Furthermore, we show that Epac induces cardiomyocyte hypertrophy. This process is associated with the activation of Rac and the calcineurin/NFAT signaling pathway, which coordinately regulates cell growth and gene expression. Altogether, these findings identify the cAMP-binding protein, Epac, as a new positive regulator of cardiac growth.

**Materials and Methods**

**Adenoviral Infection**

Bicistronic adenoviruses (Ad5) bearing either Epac WT or Epac cAMP under the control of a cytomegalovirus promoter and green fluorescent protein (GFP) under internal ribosomal entry site control were constructed and amplified at the Genethon Center of Evry (France). Adenoviruses encoding VIVIT, a selective peptide inhibitor of calcineurin-mediated NFAT activation, and Rac were provided by Drs S. Kraner and C. Norris (University of Kentucky) and T. Finkel (Cardiology Branch, National Heart, NIH, Bethesda, Md), respectively. One day after plating, cardiomyocytes were incubated for 2 hours with recombinant adenoviruses. After removal of the virus suspension, cells were replaced in maintenance medium for 2 days and then stimulated with the different drugs. Viruses were used at a multiplicity of infection (MOI) of 100.

**Plasmid Constructs and Transfection**

The plasmid constructs were generously provided by the following: the rat ANF promoter fused to the luciferase reporter gene (ANF-Luc) by Dr K. Knowlton, Luciferase reporter genes linked to promoters for skeletal muscle α-actin (SkM-α-actin-Luc) and serum response element-regulated c-fos (c-fos-SRE-Luc) by Dr M. D. Schneider, Epac1 plasmid constructs by Drs J. Bos and X. Cheng. The luciferase reporter plasmid driven by four NFAT consensus binding sites (NFAT-Luc) was obtained from Stratagene. Transient transfection experiments were performed with Lipofectamine 2000 (Invitrogen Life Technologies, France) in optimum medium in the presence of 1 μg of the various plasmid constructs according to the manufacturers’ instructions.

**Rac Activation Assay**

Rac pull-down experiments were performed using a GST fusion protein containing the Cdc42/Rac Interactive Binding Domain (CRIB) of p21-activated kinase (PAK) exactly as previously described.11

**Statistical Analysis**

Results are expressed as means±SEM. Differences between groups have been analyzed by one-way ANOVA followed by unpaired Student t test. Differences were considered significant at P<0.05, P<0.01, and P<0.001.

For a description of other methods, see the expanded Materials and Methods, available online at http://circres.ahajournals.org.

**Results**

**Epac Activates the Small G Protein Rac in Cardiomyocytes**

Activation of endogenous Epac with a selective activator of this GEF, 8-pCPT-2′,O-Me-cAMP (8-CPT), which does not activate PKA17 increased Rac activation in rat cardiac myocytes (Figure 1). Similarly, infection of cardiomyocytes with an adenovirus encoding Epac WT (Ad.Epac WT) significantly enhanced Rac GTP-loading compared with control cells infected with GFP (Figure 1). Rac activation was further increased when cells infected with Ad.Epac WT were treated with 8-CPT (Figure 1). An adenovirus bearing a constitutive activated form of Epac1 (Ad.Epac ΔcAMP)2 also induced Rac activation (Figure 1). Altogether, these results demonstrate that recombinant and native Epac increase the amount of Rac-GTP in cardiomyocytes.
Epac Increases the Expression of Hypertrophy Gene Markers

As Rac has been found to be involved in cardiac myocyte hypertrophy,15,16 we next tested the potential involvement of Epac in this process. Re-expression of embryonic genes and transient activation of immediate early genes are frequently used indexes of myocyte hypertrophy.18 The ability of Epac to stimulate gene expression was determined using luciferase (Luc) constructs under the control of promoters for ANF, SkM α-actin, and the c-fos-SRE. Figure 2A shows a three-fold activation of the ANF-Luc reporter gene in neonatal cardiomyocytes stimulated with 8-CPT compared with control cells. Transient transfection of EpacWT or Epac-ΔAMP increased the basal level of ANF-Luc activity (Figure 2A). The effect of EpacWT on ANF-Luc activity was further increased by the application of 8-CPT (Figure 2A). RacG12V mimicked the effect of Epac on ANF-Luc activity (Figure 2A). Next, to analyze the effect of Epac on ANF mRNA content in cardiac myocytes, we used Ad.EpacWT to maximize the expression of this GEF in primary cardiomyocyte. Consistent with the effect of Epac on ANF-Luc reporter gene, endogenous expression of ANF mRNA was significantly increased in cardiomyocytes infected with Ad.EpacWT and stimulated or not with 8-CPT, as compared with control cells (Figure 2B). Similar results were obtained with an adenovirus expressing RacG12V (Ad.RacG12V) (Figure 2B). In addition, when cotransfection experiments were performed with SkM-α-actin-Luc or c-fos-SRE-Luc, 8-CPT, EpacWT, Epac-ΔAMP, or RacG12V significantly increased Luc activity compared with control cells (Figure 2C and 2D). The effect of EpacWT on Luc activity was further increased by the application of 8-CPT (Figure 2C and 2D).

Epac Increases Cardiomyocyte Size and Sarcomeric Organization

Further studies were undertaken to determine the effects of Epac on other features of the hypertrophic program such as cell size and sarcomeric organization. Cardiomyocyte treatment with Ad.GFP and 8-CPT as well as infection of cardiomyocytes with Ad.EpacWT induced an apparent increase of the F-actin meshwork and a heavily striated appearance, reflecting the organization of this F-actin cytoskeleton into sarcomeric structures, as compared with cardiomyocytes infected with Ad.GFP alone (Figure 3A). Cells overexpressing Epac were hypertrophied and were not contaminated by fibroblast as shown by the α-actinin staining in supplementary Figure I. In addition, the effects of Epac on sarcomeric organization were comparable to Ad.Epac-ΔAMP (data not shown) and PE (Figure 3A), a well-known inducer of cardiac hypertrophy.

Next, we measured the effect of Epac on cell surface area. Activation of endogenous Epac with 8-CPT produced a two-fold increase in cell surface area when compared with cells infected with control Ad.GFP (Figure 3B). Identical results were obtained when cardiomyocytes were infected with Ad.EpacWT (Figure 3B), Ad.Epac-ΔAMP (data not shown), or Ad.GFP and treated with PE (Figure 3B). The effect of Ad.EpacWT on cell surface area was not further increased in the presence of 8-CPT suggesting that basal intracellular cAMP was sufficient to activate recombinant Epac to induce its maximal effect on protein synthesis (Figure 3B). Finally, the effect of Epac on protein synthesis was analyzed by measurement of [3H]-leucine incorporation into cardiac myocytes. Expression of this cAMP-GEF resulted in an increase in [3H]-leucine uptake into cardiomyocytes (Figure 3C). Similarly, cell treatment with 8-CPT or the gold standard, PE resulted in an approximately two-fold increase in protein synthesis (Figure 3C). Altogether, these results show that Epac activation confers to primary cardiomyocytes all the features of the hypertrophic phenotype.

Intracellular Ca^{2+} Is Involved in Epac-Dependent Rac Activation

Alterations in intracellular Ca^{2+} handling progressively exacerbates a hypertrophic or cardiomyopathic phenotype, in part
through sustained activation of Ca\(^{2+}\)-sensitive signal transduction pathways.\(^{19}\) Given the involvement of Epac in cardiac hypertrophy, we examined whether its activation could affect intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in neonatal myocytes (Figure 4). At physiological external [Ca\(^{2+}\)], cardiac myocytes exhibited spontaneous Ca\(^{2+}\) transients with a low frequency (0.120 \pm 0.015 Hz, n = 20) (Figure 4A). Application of the Epac agonist 8-CPT triggered a dramatic increase in the frequency of these Ca\(^{2+}\) oscillations (0.51 \pm 0.04 Hz, n = 7) without changing the amplitude of the spikes. This effect was also observed at 100 nM 8-CPT (0.40 \pm 0.05 Hz, n = 13, data not shown).

Because Epac induced Rac activation, we examined the dependence of Rac activation on Ca\(^{2+}\) signaling. Treatment of cardiac myocytes with the Ca\(^{2+}\) ionophore ionomycin as well as an inhibitor of the Ca\(^{2+}\)-ATPase, thapsigargin increased Rac activation in a time dependent manner (Figure 4B and 4C). The effect of ionomycin and thapsigargin on Rac activation was as potent as the positive control, PE (Figure 4B and 4C). Pretreatment with BAPTA-AM, an intracellular Ca\(^{2+}\) chelator, attenuated Epac-induced Rac activation (Figure 4D). From these results we conclude that elevation of intracellular [Ca\(^{2+}\)], after Epac activation is sufficient to activate Rac.
Epac Activates the Hypertrophic Calcineurin/NFAT Signaling Pathways

One prominent Ca^{2+}-dependent pathway that plays a crucial role in cardiomyocyte hypertrophy involves the phosphatase calcineurin. Activation of calcineurin by Ca^{2+} results in the dephosphorylation and nuclear translocation of cytoplasmic NFAT transcription factors, which then upregulate transcription of hypertrophic genes. To test whether endogenous Epac may activate the hypertrophic calcineurin NFAT signaling pathway, cardiomyocytes were transfected with NFAT-Luc and treated or not with 8-CPT (Figure 5A). 8-CPT significantly increased NFAT transcriptional activity as compared with control cells (Figure 5A). Accordingly, 8-CPT increased NFAT nuclear translocation (supplementary Figure II). The stimulating effect of 8-CPT on NFAT-Luc was significantly blocked by a pharmacological inhibitor of calcineurin, cyclosporine A (CsA) or transfection of a dominant negative form of Epac (Epac^{WT} to 148) (Figure 5A).

Recombinant Epac^{WT} also increased NFAT transcriptional activity which was blocked by CsA or an adenovirus bearing a selective peptide inhibitor of calcineurin named VIVIT (Ad.VIVIT) (Figure 5B and 5C). Consistent with these findings, cardiac myocytes infected with Ad.Epac^{WT} and treated or not with 8-CPT (Figure 5D), or Ad.EpacΔAMP (data not shown) had an increased content of mRNA encoding the modulatory calcineurin-interacting protein 1 (MCIP1), a mediator of calcineurin signaling during cardiac hypertrophy. Furthermore, coinfection with Ad.VIVIT and Ad.Epac^{WT} reduced the enhancement of sarcomeric organization and cell surface area induced by Ad.Epac^{WT} (Figure 6A and 6B). Altogether these data show that NFAT is a downstream component of Epac hypertrophic signaling pathway.

Figure 4. Intracellular Ca^{2+} is involved in Epac-dependent Rac activation. A, Effect of 8-CPT (10^{-5} mol/L) on spontaneous spiking activity at 1.8 × 10^{-3} mol/L external [Ca^{2+}]. Cardiomyocytes at day 1 or 2 after plating were loaded with the Ca^{2+} indicator Fluo3-AM and perfused with a control external Ringer solution. B to D, Effect of ionomycin, thapsigargin and BAPTA-AM on Rac activation. Ventricular cardiomyocytes were treated at 2 days in vitro with either ionomycin (10^{-6} mol/L) (B) or thapsigargin (0.2 × 10^{-6} mol/L) (C) for different times of incubation or PE (10^{-5} mol/L) for 15 minutes. D, Cells were pretreated with BAPTA-AM (1.5 × 10^{-5} mol/L) for 10 minutes and then they were stimulated with 8-CPT (10^{-5} mol/L) for 15 minutes. The amount of Rac-GTP was determined by pull-down experiments.
Discussion

The present study shows for the first time that cAMP-dependent activation of Epac induces cardiomyocyte hypertrophy. This is based on our observation that Epac activation leads to morphological changes, cytoskeletal reorganization, increases in protein synthesis, and induces expression of cardiac hypertrophic markers. In addition, we found that Epac activates a prohypertrophic signaling pathway which involves calcineurin and its primary downstream effector, NFAT. Epac-induced NFAT activation was dependent on Rac activity. Interestingly, overexpression of Epac was able to influence cardiomyocyte morphology without cAMP analogue treatment indicating that the level of intracellular cAMP was sufficient to activate the recombinant EpacWT protein. Similar observations have been reported in other cellular systems because transfection of EpacWT in HEK293 and COS cells has been previously shown to influence cell signaling and morphology at resting basal cAMP levels.4 Theses findings suggest that this cAMP-GEF may be localized in subcellular membrane fractions of cardiomyocytes where intracellular cAMP concentration is high. Further experiments are required to determine the precise localization of Epac in cardiac myocytes and its association with proteins (ie, phosphodiesterases) that regulate cAMP gradient formation.

In our study, we showed that the Epac-specific cAMP analogue 8-CPT produces bursts of Ca\(^{2+}\)/H\(_{11001}\) transients in newborn cardiac myocytes. Our findings are in line with recent studies in pancreatic \(\beta\)-cells and INS-1 insulin-secreting cells, demonstrating a Epac-dependent mobilization of intracellular Ca\(^{2+}\) by the cAMP-elevating hormone glucagon-like peptide 1 and the implication of Epac in this effect.25 In these cells, activation of endogenous Epac triggers Ca\(^{2+}\)-induced Ca\(^{2+}\) release26 and it is suggested that a functional coupling exists between Epac and the RyR in these cellular systems.27 Interestingly, the small GTPase Rap1 which is an effector of Epac is suspected to play a role in cAMP-induced [Ca\(^{2+}\)]\(_{i}\) increase via SERCA3b in megakaryocytes.28,29 Therefore,
one could imagine in cardiac myocytes, that Epac might interact with Ca\(^{2+}\) release channels. Such hypothesis is currently undergoing investigation.

We found that Epac induced Rac activation in primary cardiomyocytes. This is in accordance with our recent findings showing that Epac induces Rac activation in a cAMP-dependent but PKA-independent manner in noncardiac cells such as primary cortical neurons and CHO cells.\(^{11}\) Because we found that Rac was activated by Ca\(^{2+}\) on Epac stimulation, it is reasonable to think that Rac might be regulated by a GEF, which is sensitive to Ca\(^{2+}\). Such a GEF has been reported for small GTPases of the Ras family.\(^{30,31}\) Another molecular target which could be involved in Ca\(^{2+}\)-dependent Rac activation is the Rho GDP-dissociation inhibitor (RhoGDI). RhoGDI retains Rac into the cytoplasm and must dissociate to allow Rac to encounter its GEFs.\(^{32,33}\) Recently, Price et al\(^{34}\) have shown that Ca\(^{2+}\) induces a disruption of the Rac-RhoGDI complex leading to the translocation and activation of Rac in PC3 cells. Thus, one could speculate that such a mechanism might occur in cardiomyocytes and contribute to Epac-induced Ca\(^{2+}\)-dependent Rac activation.

We report for the first time to our knowledge that Epac is implicated in the activation of NFAT in cardiac myocytes. The ability of Epac to stimulate NFAT activity was significantly inhibited by treatment with CsA and VIVIT, suggesting that calcineurin activity is regulated by Epac. Accordingly, we found that Epac upregulates the expression of MCIP1, a well known modulator of calcineurin signaling that possesses a series of NFAT binding sites in its gene promoter.\(^{23,35}\) In addition, Ad.VIVIT partially reversed Epac-induced cardiomyocyte hypertrophy indicating that Epac is a new regulator of the hypertrophic calcineurin/NFAT signaling pathway. As the Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) is well-known to play a key role in cardiac hypertrophy,\(^{36}\) it would be interesting to test the potential involvement of this signaling pathway in Epac-induced cardiac hypertrophy. In addition, Epac might be an important mediator of oxidative stress because an antioxidant blocked its effect on ANF-Luc. In accordance with this observation, Rac activation is thought to be an important mediator of ROS production induced by adrenoreceptor stimulation.\(^{37}\) Furthermore, G\(_{\text{q/11}}\)-mediated ROS production is essential for angiotensin II-induced NFAT transcriptional activation.\(^{24}\)

An important finding of the present study is that the effect of Epac on NFAT activation was inhibited by Rac\(^{G17N}\), a negative dominant form of Rac. Inversely, Rac under its activated form increased NFAT activity and this effect was blocked by CsA. In line with these data, we found that Rac\(^{G17N}\) inhibited Epac-induced ANF transcriptional activity and cell growth. Altogether these results indicate that Rac is involved in the regulation of the hypertrophic calcineurin/NFAT signaling pathway initiated by Epac in cardiomyocytes. In contrast to our findings, a previous study has shown that Ras but not Rho GTPases regulates NFAT activity in cardiac cells.\(^{38}\) The reasons for these discrepancies are still unclear. However, the stimulating effect of Rac on NFAT activity is supported by previous reports showing that humoral factors induce Rac-dependent NFAT activation in various cellular systems including immune and cardiac cells.\(^{24,39,40}\) In addition, Rac\(^{G12V}\) has been shown to upregulate ANF expression in rat primary cardiac myocytes.\(^{14}\)

Besides Epac, sustained activation of other cAMP effectors have been shown to be deleterious for cardiac cells. For instance, constitutive activation of PKA in the hearts of transgenic mice leads to cardiomyocyte hypertrophy and a progressive decline in cardiac function.\(^{41}\) In a similar manner, increasing \(\beta_1\)-adrenergic receptor (\(\beta_1\)-AR) signaling cascade or G\(_{\text{as}}\) protein levels induces, through intracellular Ca\(^{2+}\) elevation, a progressive development of cardiac hypertrophy and heart failure.\(^{42,43}\) But although our data and the these observations point to a negative role of persistent activity of

Figure 6. Ad.VIVIT inhibits Epac-induced cardiomyocyte hypertrophy. A. Fluorescent microscopic analyses of the effects of Epac on sarcomeric organization. Morphology of representative myocytes 48 hours after infection with Ad.GFP (control), Ad.VIVIT, Ad.Epac\(^{WT}\), or Ad.Epac\(^{WT}\) and Ad.VIVIT is shown. B. Photographic images of cardiac myocytes infected as above were digitized. Areas (10\(^{-5}\) m\(^2\)) of around 50 individualized cells per condition from 3 independent experiments were determined by computer-assisted planimetry. Values show the means±SEM. *\(P<0.05\), **\(P<0.01\), and ***\(P<0.001\) compared with control or vs indicated values.
cAMP/Epac/PKA, any elevation of cAMP does not automatically cause deleterious effects. For instance, transgenic mice overexpressing β2-AR in the heart,44 Adenyl cyclase type 6 (AC6)45 or AC846 do not show early signs of hypertrophy or heart failure. Clearly, these data show that the same second messenger conveys different information and cAMP compartmentation is a key actor and determines the quality of the response. As a next step, it therefore will be crucial to determine not only the spatial localization of Epac and its possible interaction with cAMP-PDE but also the neurohor-
monal factors which are involved in the regulation of its activity.

Thus, we propose a new cAMP signaling pathway in which activation of Epac leads to an increase in [Ca^{2+}], which then activates calcineurin and Rac. The latter controls NFAT activation. This signaling cascade activates hypertrophic gene expression and induces the morphological aspects of cardiac myocyte hypertrophy. Our results thus open new insights into the signaling pathways by which cAMP may mediate its biological effects in cardiomyocytes.

Figure 7. Involvement of Rac in Epac-induced NFAT-dependent cardiomyocyte hypertrophy. A to C, Cardiomyocytes infected with Ad.GFP (control), Ad.RacS17N, Ad.EpacWT, or Ad.EpacWT, and Ad.RacS17N were transfected with either NFAT-Luc or ANF-Luc. Two days after, Luc activity was determined. Values are means±SEM of at least 3 separate experiments performed in triplicate. D and E, Ad.RacS17N reverses Epac-induced cardiomyocyte hypertrophy. D, Photographic images of cells infected for 2 days with Ad.GFP (control), Ad.EpacWT, Ad.RacS17N, or Ad.EpacWT, and Ad.RacS17N were digitized. E, The areas (10^5 m^2) of 50 individualized cells per condition from 3 independent experiments were determined by computer-assisted planimetry. Values show the means±SEM. *P<0.05, **P<0.01, and ***P<0.001 vs control cells or indicated values.
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References


