

The cAMP binding protein Epac regulates cardiac myofilament function

Olivier Cazorla^{a,b}, Alexandre Lucas^{c,d}, Florence Poirier^d, Alain Lacampagne^{a,b,1}, and Frank Lezoualc'h^{c,d,1}

^aInstitut National de la Santé et de la Recherche Médicale, U637, Physiopathologie Cardiovasculaire; 34295 Montpellier, France; ^bUniversité Montpellier 1, IFR3, 35295 Montpellier, France; ^cInstitut National de la Santé et de la Recherche Médicale, UMR-S 769, Signalisation et Physiopathologie Cardiaque, 92296 Châtenay-Malabry, France; and ^dUniversité Paris-Sud, Faculté de Pharmacie, IFR141, 92296 Châtenay-Malabry, France

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In the heart, cAMP is a key regulator of excitation–contraction coupling and its biological effects are mainly associated with the activity of protein kinase A (PKA). The aim of this study was to investigate the contribution of the cAMP-binding protein Epac (Exchange protein directly activated by cAMP) in the regulation of the contractile properties of rat ventricular cardiac myocytes. We report that both PKA and Epac increased cardiac sarcomere contraction but through opposite mechanisms. Differently from PKA, selective Epac activation by the cAMP analog 8-(4-chlorophenylthio)-2'-O-methyl-cAMP (8-pCPT) reduced Ca²⁺ transient amplitude and increased cell shortening in intact cardiomyocytes and myofilament Ca²⁺ sensitivity in permeabilized cardiomyocytes. Moreover, ventricular myocytes, which were infected *in vivo* with a constitutively active form of Epac, showed enhanced myofilament Ca²⁺ sensitivity compared to control cells infected with green fluorescent protein (GFP) alone. At the molecular level, Epac increased phosphorylation of 2 key sarcomeric proteins, cardiac Troponin I (cTnI) and cardiac Myosin Binding Protein-C (cMyBP-C). The effects of Epac activation on myofilament Ca²⁺ sensitivity and on cTnI and cMyBP-C phosphorylation were independent of PKA and were blocked by protein kinase C (PKC) and Ca²⁺ calmodulin kinase II (CaMKII) inhibitors. Altogether these findings identify Epac as a new regulator of myofilament function.

calmodulin kinase II | contraction | exchange protein activated by cyclic AMP | sarcomeric proteins | protein kinase C

The second messenger cAMP is a key mediator of the sympathetic system and is involved in the control of cardiac function. Besides the cyclic nucleotide pacemaker channel, cAMP acts through the serine/threonine-specific protein kinase A (PKA) to modulate cardiac contractility via intracellular Ca²⁺ movements (1). Ca²⁺ is essential for cardiac electrical activity and directly activates myofilaments, thus inducing their contraction. In cardiac myocytes, PKA targets various Ca²⁺ handling proteins involved in excitation–contraction (EC) coupling, such as the sarcolemmal L-type Ca²⁺ channel and the sarcoplasmic reticulum (SR) ryanodine receptor (RYR) (1). The effect of PKA on myofilament protein phosphorylation is also critical for cardiac dynamics and contractility (2). For instance, under β -adrenergic stimulation, PKA-dependent phosphorylation of the thin filament protein cardiac Troponin I (cTnI) results in reduction of myofilament Ca²⁺ sensitivity and increase of cross-bridge cycling rate, leading to acceleration of relaxation (3). Phosphorylation of the thick filament protein cardiac Myosin Binding Protein-C (cMyBP-C) by PKA appears to affect actin and myosin interactions (4) and contributes to PKA effects on Ca²⁺ sensitivity (5). In addition, PKA-dependent phosphorylation of Titin has been shown to reduce cardiomyocyte stiffness and consequently heart diastolic force (6).

A decade ago, a family of proteins directly activated by cAMP was discovered, adding another layer of complexity to the cAMP-mediated signaling cascade (7, 8). These proteins, named Epac (Exchange proteins directly activated by cAMP), are guanine nucleotide exchange factors (GEFs) for Rap1 and Rap2

small GTPases (9). Two variants of Epac exist (Epac1 and Epac2), both of which are activated by physiologically relevant concentrations of cAMP (9). Epac1 is highly expressed in the heart and displays comparable affinity for cAMP as a PKA holoenzyme (8, 10). With the recent availability of a selective Epac activator, the cAMP analog 8-(4-chlorophenylthio)-2'-O-methyl-cAMP (8-pCPT) (11), several studies have revealed the critical role of Epac in various cellular processes such as cell permeability and cardiomyocyte hypertrophy (12–14). Interestingly, recent evidence indicates that Epac activation alters Ca²⁺ signaling in the SR (15, 16). However, the role of Epac in the regulation of cardiomyocyte contractility is still unknown.

Here we report that Epac potentiates cardiac contraction despite a decrease in the amplitude of Ca²⁺ transient. We show that specific activation of Epac or overexpression of a constitutively active form of Epac increases myofilament Ca²⁺ sensitivity in permeabilized ventricular cardiac myocytes in a PKA-independent manner. This is correlated with an increase in phosphorylation of cMyBP-C and cTnI. In addition, we report that Epac-dependent effects on myofilament proteins involve both protein kinase C (PKC) and Ca²⁺ Calmodulin-Kinase II (CaMKII). Taken together our data show that independently of its effect on SR function, Epac has a direct effect on the contractile machinery and is a new piece of the regulatory cascade of cardiac contractile function.

Results

Epac Regulates Myofilament Ca²⁺ Sensitivity in a PKA-Independent Manner. To test the effect of Epac activation on cell contraction, we recorded simultaneously changes in sarcomere length (SL) and intracellular Ca²⁺ in indo-1-loaded intact cardiomyocytes stimulated at 1 Hz prior to and during treatment with the Epac selective activator 8-pCPT (1 μ M) (Fig. 1A). SL shortening started to increase progressively \approx 1 min after addition of 8-pCPT to reach a plateau within 5 min. 8-pCPT increased SL shortening and decreased calcium transient in a concentration-dependent manner (Fig. 1B and C). The steady-state maximal inhibition of Ca²⁺ transient could not be determined because arrhythmic events occurred at high concentrations (starting at 1 μ M), probably because of the increase in the diastolic Ca²⁺ level (Fig. 1D). The gain of function (SL shortening–Ca²⁺ transient ratio) that is a good indicator of the myofilament Ca²⁺ sensitivity increased from 0.1 to 1 μ M 8-pCPT (Fig. 1E). The speed of SL shortening increased [supporting information (SI) Fig. S1A] and diastolic SL decreased (Fig. S1C), both in a concentration-

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¹To whom correspondence may be addressed. E-mail: alain.lacampagne@inserm.fr or frank.lezoualc'h@u-psud.fr.

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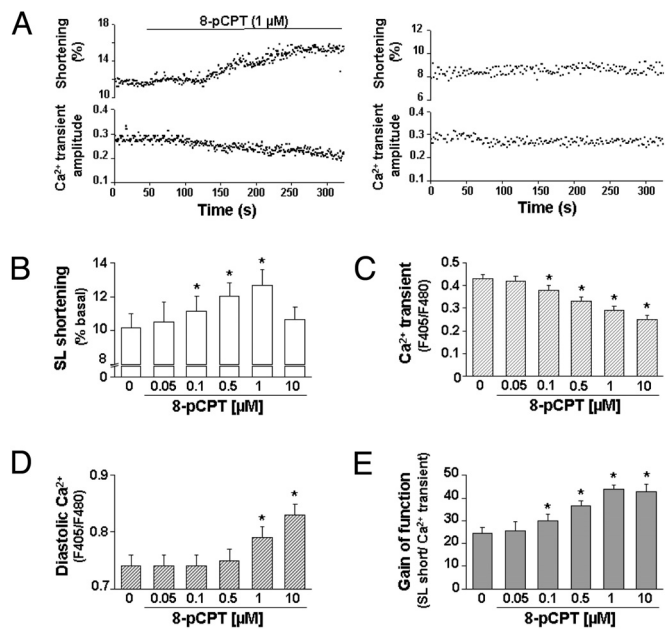


Fig. 1. Epac regulates the contractile machinery. (A) Effect of 8-pCPT (1 μM) perfusion on sarcomere shortening (Left, Upper) and intracellular calcium transient amplitude (Left, Lower) in intact ventricular cardiomyocytes stimulated at 1 Hz. Activation of Epac increased progressively SL shortening and reduced the amplitude of Ca²⁺ transient. The maximal effect was observed within 5 min. Right, a time control of SL shortening and calcium transient. (B–E) Concentration-dependent effect of 8-pCPT on SL shortening (B), Ca²⁺ transient amplitude (C), diastolic calcium level (D), and the gain of function (E), which corresponds to the ratio between SL shortening and calcium transient amplitudes ($n = 11$ cells). *, $P < 0.05$ versus control.

dependent manner. The speed of SL relaxation tended to increase with 8-pCPT but the differences did not reach significance (Fig. S1B). Altogether, these data suggest a dual effect of Epac activation on SR Ca²⁺ signaling (i.e., Ca²⁺ basal level and transient amplitude) and a direct effect on the contractile machinery (SL shortening).

To determine the effect of Epac activation on the contractile machinery properties, we measured the relationship between Ca²⁺-activated tension and internal concentration of Ca²⁺ expressed as pCa ($= -\log[\text{Ca}^{2+}]$) in permeabilized cardiomyocytes (Fig. 2A). Permeabilized cardiomyocytes allow us to study the properties of the contractile machinery and its relation to calcium independently of the amount released by SR (4, 5, 17, 18). After incubation with 8-pCPT (1 μM), the curve representing the tension–pCa relationship was significantly shifted to the left (i.e., increase in pCa₅₀), indicating that for a given amount of Ca²⁺, more force was produced by the myofilaments upon Epac activation. The other contractile parameters such as maximal active tension, passive tension, and the Hill coefficient were not affected by Epac activation (Table S1). Conversely, permeabilized cells incubated with either a recombinant catalytic subunit of PKA (200 UI) or 6-BnZ-cAMP (200 μM), a selective and membrane-permeant activator of PKA, showed a significant decrease in pCa₅₀ (Fig. 2A, Fig. S2). These data demonstrate that Epac and PKA have opposite effects on Ca²⁺-activated force and myofilament Ca²⁺ sensitivity (Fig. 2A, Fig. S2). Similarly, we found that ventricular myocytes isolated from myocardial tissues infected with a bicistronic adenovirus bearing a constitutively active form of Epac1 and green fluorescent protein (GFP) (Ad-Epac^{ΔcAMP}) showed an increase in pCa₅₀ (Fig. 2B). The effect of Epac^{ΔcAMP} on pCa₅₀ was similar to the effect obtained with 8-pCPT (1 μM) and was not further increased by addition of this drug (Fig. 2B). Finally, we found that Epac effect on

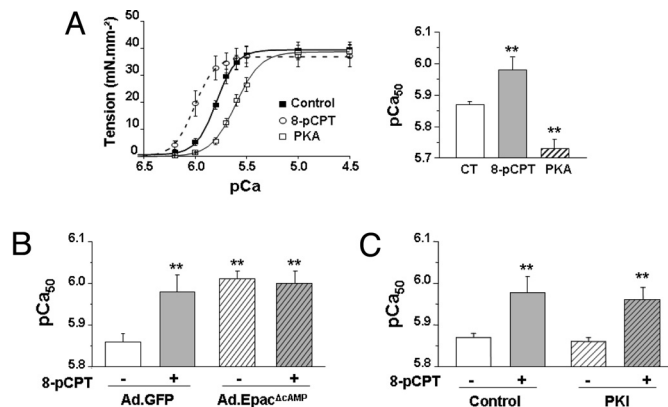


Fig. 2. Epac regulates myofilament Ca²⁺ sensitivity in a PKA-independent manner. (A) The relationship between Ca²⁺-activated tension and intracellular Ca²⁺ content was measured in isolated, permeabilized cardiomyocytes at 2.3 μM SL. The relationship was fitted with a modified Hill equation and the pCa at which half of the maximal tension is developed (pCa₅₀) was determined as an index of myofilament Ca²⁺ sensitivity (see SI Materials and Methods for more details). Preincubation of cells with 8-pCPT (1 μM for 10 min) increased myofilament Ca²⁺ sensitivity as indicated by the shift toward the left of the curve and the increase in pCa₅₀ ($n = 14$ cells). In similar conditions, recombinant PKA catalytic subunit induced an opposite effect that reflects desensitization of the myofilaments ($n = 10$ cells). (B) Constitutively active Epac (Epac^{ΔcAMP}) increased myofilament Ca²⁺ sensitivity as indexed by pCa₅₀. The left ventricular free wall of rats was infected with adenoviruses encoding GFP (Ad-GFP) (control) or bicistronic adenoviruses coexpressing GFP and Epac^{ΔcAMP} (Ad-Epac^{ΔcAMP}). Three days later, cells were isolated and myofilament Ca²⁺ sensitivity of permeabilized ventricular cardiomyocytes expressing GFP was measured after incubation, or not, with 8-pCPT (1 μM) for 10 min. Ad-GFP, $n = 10$; Ad-Epac^{ΔcAMP}, $n = 16$ cells (3 rats per condition). (C) Effect of PKI, a PKA inhibitor, on 8-pCPT-induced myofilament Ca²⁺ sensitization. Myofilament Ca²⁺ sensitivity (pCa₅₀) was determined in permeabilized adult ventricular cardiomyocytes treated, or not, with 8-pCPT (1 μM) for 10 min and in the presence or absence of PKI (5 μM); $n = 16$ cells (3 rats). Results are expressed as means ± SEM. **, $P < 0.01$ compared with nontreated cells.

myofilament Ca²⁺ sensitivity was independent of PKA because a PKA inhibitor, PKI (5 μM), failed to inhibit the effect of 8-pCPT on pCa₅₀ (Fig. 2C). Altogether, our data show that Epac activation increases myofilament Ca²⁺ sensitivity in a PKA-independent manner in adult ventricular cardiac myocytes.

Epac Regulates Phosphorylation of cMyBP-C and cTnI. Myofilament Ca²⁺ sensitivity is regulated through phosphorylation of sarcomeric proteins, such as cTnI and cMyBP-C, which are known to mediate myocardial responses to cAMP via PKA (3). Therefore we checked whether activated Epac could also regulate the phosphorylation of such proteins. Indeed, activation of endogenous Epac by 8-pCPT (1 μM) increased phosphorylation of cMyBP-C at Ser²⁸² (P-cMyBP-C) in freshly isolated adult cardiomyocytes (Fig. 3A). The level of P-cMyBP-C following Epac activation was comparable to that observed in cells treated with isoproterenol (ISO) (100 nM), the standard β-adrenergic receptor agonist (Fig. 3A). Similarly, infection of cardiomyocytes with adenoviruses encoding the wild-type form of human Epac1 (Ad-Epac^{WT}) induced a partial increase in P-cMyBP-C that was further enhanced by treatment with 8-pCPT (1 μM) (Fig. 3B). In myocytes expressing Ad-Epac^{ΔcAMP}, the P-cMyBP-C level was comparable to that obtained in myocytes infected with Ad-Epac^{WT} and treated with 8-pCPT (Fig. 3B). In addition, Epac-induced P-cMyBP-C was independent of PKA, because PKI failed to block the effect of 8-pCPT on P-cMyBP-C phosphorylation (Fig. 3C). As previously reported, cMyBP-C was phosphorylated by PKA after ISO stimulation (Fig. 3D); however, ISO-induced cMyBP-C phosphorylation was only partially re-

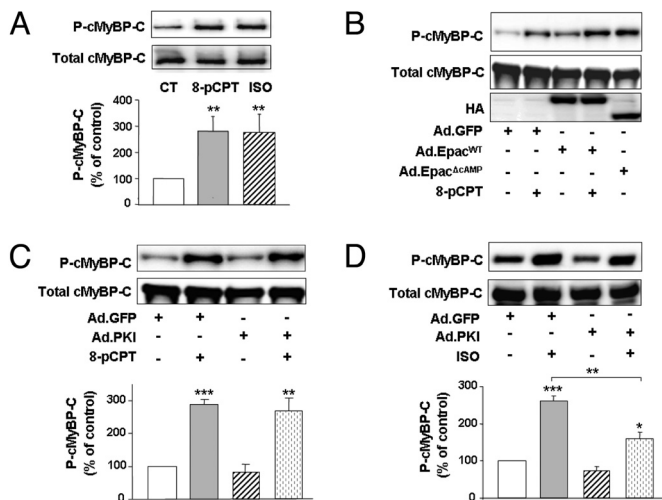


Fig. 3. Epac regulates phosphorylation of the thick filament protein cMyBP-C. (A) Effect of 8-pCPT (1 μ M) or ISO (100 nM) on cMyBP-C phosphorylation (P-cMyBP-C) in freshly isolated adult cardiomyocytes. Cells were incubated with the drugs for 10 min and P-cMyBP-C was determined by Western blotting using an anti-P-cMyBP-C (P-Ser²⁸²) antibody as described in *SI Materials and Methods*. Membranes were then stripped and probed for total cMyBP-C expression to confirm equivalent protein loading. Lower, immunoblots were quantified and data were normalized to total cMyBP-C expression. (B and C) Effect of 8-pCPT (1 μ M) and (D) effect of ISO (100 nM) on P-cMyBP-C in adult cardiomyocytes infected with Ad-GFP, Ad-Epac^{WT}, Ad-Epac^{ΔcAMP}, or Ad-PKI (PKA inhibitor). Immunoblots against P-cMyBP-C and total cMyBP-C were performed as in A. (B) An anti-HA antibody was used to monitor the expression levels of Epac^{WT} and Epac^{ΔcAMP}. (C and D) Immunoblots were quantified and data normalized to total cMyBP-C. Results are means \pm SEM from 6 (A) or 4 (B–D) independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared with control or indicated values. CT, control.

duced by treatment with PKI, suggesting that Epac contributes to the regulation of cMyBP-C phosphorylation induced by β -adrenergic receptors (β -AR) (Fig. 3D). These results may explain the remaining increase in intact cell shortening after ISO stimulation in the presence of a PKA inhibitor, KT5720 (Fig. S1D). Accordingly, myocytes treated with ISO (100 nM) and infected with a short hairpin RNA targeting Epac1 (Ad-shEpac) to knock down its expression showed a decreased level of P-cMyBP-C compared to cells infected with shRNA sequence control (Ad-shCT) and stimulated with ISO (Fig. S3A and B). Knockdown of Epac1 slightly decreased pCa₅₀ in basal condition but this was not statistically significant as compared to Ad-shCT control cells (Fig. S3C). ISO decreased myofilament Ca²⁺ sensitivity in a similar fashion in Ad-shCT and Ad-shEpac cells (Fig. S3C). Importantly, 8-pCPT failed to increase myofilament Ca²⁺ sensitivity in cells infected with Ad-shEpac, indicating that Epac specifically regulates the contractile properties of cardiomyocytes (Fig. S3C).

We next investigated whether Epac activation could also regulate phosphorylation of cTnI. Differently from ISO, 8-pCPT failed to induce cTnI phosphorylation at Ser^{22/23} even in cells infected with Ad-Epac^{WT} or Ad-Epac^{ΔcAMP} (Fig. 4A). To determine whether Epac activation could influence cTnI phosphorylation at other sites, myofilament proteins were extracted from myocytes infected with either Ad-GFP or Ad-Epac^{WT} and treated or not with 1 μ M 8-pCPT or infected with Ad-Epac^{ΔcAMP}. In extracts from cells treated with 1 μ M 8-pCPT or infected with Ad-Epac^{ΔcAMP}, 1 band of \approx 23 kDa showed a significant increase in phosphorylation, as determined with the ProQ phospho-protein stain (Fig. 4B). Tandem mass spectrometry revealed that this 23-kDa band corresponded to cTnI (MASCOT score: 196; number of identified peptides: 19). The

effect of Epac activation on cTnI phosphorylation was not abolished by PKI (Fig. 4C), which, on the other hand, completely prevented PKA-dependent phosphorylation of cTnI (Fig. S4). To confirm a change in the phosphorylation status of cTnI upon Epac activation, myocytes treated or not with 8-pCPT were processed for 2D electrophoresis and subjected to ProQ Diamond staining to reveal phosphoproteins. In an area of interest corresponding to a theoretical spot of cTnI (pI 9.57; M_r 25 kDa), we identified 5 phosphorylated spots corresponding to cTnI by tandem mass spectrometry of peptides separated by reverse phase liquid chromatography (LC/MS/MS). Fig. 4D shows that cTnI was resolved as a train of 5 spots differing in their phosphorylation level in basal condition. The intensity of the train of phosphorylation was modified in the presence of 8-pCPT with spot 5 being the most phosphorylated. Altogether these data show that Epac induces a change in the phosphorylation status of cTnI and cMyBP-C in a PKA-independent manner.

Signaling Pathways Involved in Myofilament Phosphorylation Induced by Epac.

Because the permeabilization process could induce a loss of Epac expression and its potential effectors, we first analyzed their expression in permeabilized cardiomyocytes. We found that all these proteins are present in permeabilized cardiomyocytes although some of them showed a decreased expression level as compared to intact cardiac myocytes (Fig. 5A). We next investigated the signaling pathways involved in Epac-induced myofilament phosphorylation. The primary function of Epac is to act as GEFs for Rap GTPases (9). Thus, we examined whether Rap1 was involved in the effect of Epac on myofilament phosphorylation. A Rap1 GTPase activating protein (RapGAP), which has been previously shown to abolish 8-pCPT-induced Rap1 activation in adult cardiac myocytes (19), failed to inhibit an Epac effect on P-cMyBP-C (Fig. S5). This finding demonstrates that Epac acts on myofilaments via another effector than Rap1.

Previous studies have suggested that phospholipase C (PLC) may be involved in Epac functional effects (16, 20). PLC hydrolyzes phosphatidylinositol bisphosphate (PIP2) to produce diacylglycerol (DAG) and inositol triphosphate (IP3), leading to protein PKC activation or IP3 receptor-dependent Ca²⁺ release. The stimulating effect of 8-pCPT on myofilament Ca²⁺ sensitivity was blocked by treatment with U73122, a PLC inhibitor, or calphostin-C, a PKC inhibitor (Fig. 5B). Calphostin-C also decreased cTnI and cMyBP-C phosphorylation induced by 8-pCPT (Fig. 5C and E). Because we previously identified CaMKII as an effector of Epac in cardiac myocytes (15, 19), we also evaluated whether CaMKII played a role in Epac effect on Ca²⁺ sensitivity. We found that KN-93, a pharmacological inhibitor of CaMKII, significantly inhibited the 8-pCPT effect on Ca²⁺ sensitivity in myofilaments (Fig. 5B). Consistent with these data, 8-pCPT-induced cTnI and cMyBP-C phosphorylation was blocked when myocytes were preincubated with KN-93 or infected with adenoviruses coding for a CaMKII peptide inhibitor (Ad-CaMKIIN) (Fig. 5D and F, Fig. S6). Taken together, these data show that Epac regulates myofilament Ca²⁺ sensitivity and sarcomeric protein phosphorylation through PLC-, PKC-, and CaMKII-dependent pathways.

Discussion

In this study we demonstrate that Epac regulates the contractile properties of cardiomyocytes by modulating Ca²⁺ signaling and Ca²⁺ sensitivity of sarcomeric proteins. We report that both PKA and Epac, 2 major effectors of cAMP, increase cardiac myocyte contraction but through opposite mechanisms. Indeed, Epac decreases the amount of Ca²⁺ released by the SR and sensitizes myofilaments to Ca²⁺, whereas PKA does the opposite.

Epac activation increased rapidly the rate of SL shortening and reduced significantly Ca²⁺ transient amplitude, both in a concentration-dependent manner, thus suggesting that Epac

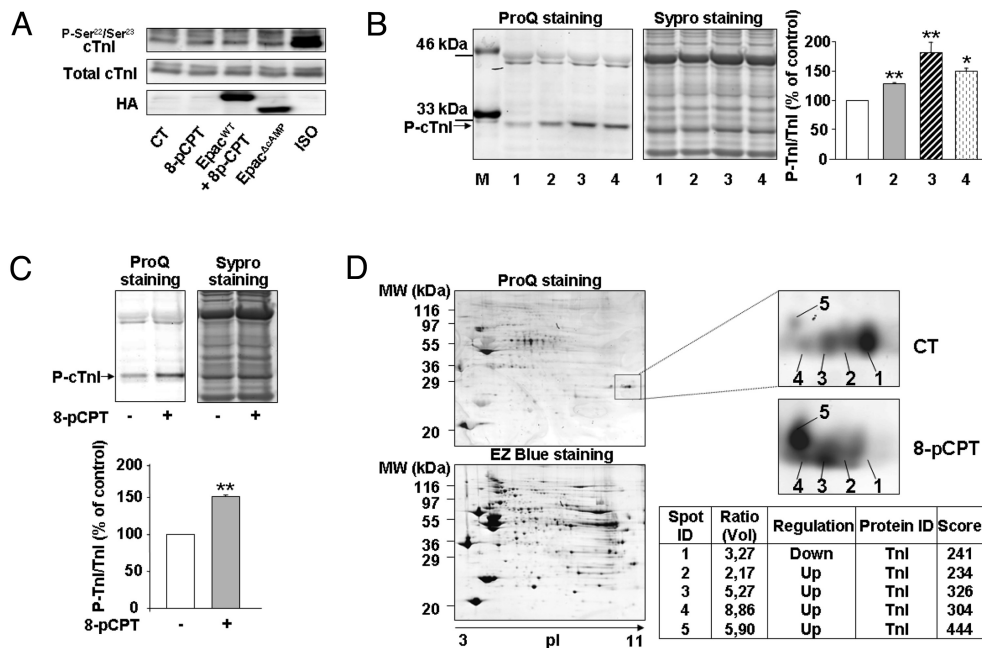


Fig. 4. Epac regulates phosphorylation of cTnI in a PKA-independent manner. (A) Representative Western blot showing cTnI phosphorylation (P-cTnI) at PKA sites (P-Ser²²/P-Ser²³) (Top). Isolated adult cardiac myocytes were infected with control Ad-GFP (CT), Ad-Epac^{WT}, or Ad-Epac^{ΔcAMP} for 36 h. Cells were then treated, or not, with 1 μM 8-pCPT or 100 nM ISO for 10 min and P-Ser²²/P-Ser²³ cTnI was determined by Western blotting as described in *SI Materials and Methods*. Total cTnI expression is shown (Middle), and anti-HA antibody was used to monitor the expression levels of Epac^{WT} and Epac^{ΔcAMP} (Bottom). (B) Adult cardiac myocytes were infected for 36 h with control Ad-GFP (CT, lane 1), Ad-GFP treated with 1 μM 8-pCPT (lane 2), Ad-Epac^{WT} treated with 1 μM 8-pCPT (lane 3), or Ad-Epac^{ΔcAMP} (lane 4). (C) Myocytes were infected with Ad-PKI and were then treated or not with 1 μM 8-pCPT for 10 min as in A and B. Protein phosphorylation was visualized with ProQ Diamond phospho-protein gel stain, followed by total protein staining with Sypro Ruby to confirm equal loading. Right, protein phosphorylation was normalized to the total protein content revealed by SYPRO Ruby staining. Results are means ± SEM of 8 (B) or 4 (C) independent experiments. *, *P* < 0.05; **, *P* < 0.01 compared with control values. Tandem mass spectrometry revealed that the phosphorylated 23-kDa band corresponded to cTnI. (D) ProQ Diamond and EZ Coomassie Blue staining of a representative 2D gel. Isolated cardiomyocytes were incubated in the absence or the presence of 8-pCPT (1 μM, 10 min). Protein extracts were separated by 2D electrophoresis using isoelectric focusing strips (18 cm, pH 3–11, nonlinear). A representative enlargement of the gel showing the marked region is illustrated. Five spots were excised and analyzed by LC/MS/MS to show the presence of cTnI. Densitometric analysis of intensities of protein spots was performed and ratios of volume values are indicated in the table. Changes in intensities of protein spots are indicated as increased (up) or decreased (down) in the stimulated 8-pCPT vs. control (CT). The 5 protein spots were identified using the MASCOT Search Engine. The score refers to the degree of similarity between a sample and a searched database match. A score of ≥65 is considered a properly identified match. ID, identity.

affects both myofilament properties and SR Ca²⁺ function independently. Similarly, we previously observed a decrease in SR Ca²⁺ load upon Epac activation in adult rat cardiac myocytes subsequent to CaMKII-dependent RyR phosphorylation and an increase in SR Ca²⁺ leak during diastole (15). These data could explain the reduced Ca²⁺ transient amplitude in the presence of 8-pCPT and the increase in diastolic Ca²⁺ level (Fig. 1C). Consistent with this finding, Curran and colleagues (21) also found that β-AR stimulation enhanced SR Ca²⁺ leak in ventricular myocytes in a CaMKII-dependent (and PKA-independent) manner. Conversely, Oestreich and colleagues (16) showed that acute treatment of single mouse ventricular cardiac myocytes with 8-pCPT increased Ca²⁺ transient amplitude in field-stimulated cells. This process was dependent on Rap1 and PLC-ε (16). The reasons for these discrepancies are unclear and may involve species differences and/or methodological approaches such as the frequency of cardiac myocyte electrical stimulation.

PKA-dependent phosphorylation of cTnI is known to reduce myofilament Ca²⁺ sensitivity and to shift the tension–pCa curve toward the right (22). This effect involves phosphorylation of cTnI at Ser²²/Ser²³ (3) and of cMyBP-C (5). Here, we report that activated Epac increases myofilament Ca²⁺ sensitivity and modulates phosphorylation of cTnI and cMyBP-C, both in a PKA-independent manner. We also show that a PLC inhibitor abolishes the stimulating effect of Epac on myofilament Ca²⁺ sensitivity, suggesting that a downstream effector of PLC is involved in Epac

signaling leading to sarcomeric protein phosphorylation. Such a candidate could be PKC because inhibition of PKC decreased 8-pCPT-induced myofilament Ca²⁺ sensitization and cTnI and cMyBP-C phosphorylation. PKC activation has been reported to potentially induce phosphorylation of cTnI at Ser^{22/23}, Ser^{43/45}, and Thr¹⁴⁴ (3). However, PKC-dependent phosphorylation of Ser^{22/23} is unlikely to be involved in an Epac effect because we did not detect increased cTnI phosphorylation at this site after Epac activation (Fig. 4A). Moreover, phosphorylation at this site would induce the same functional effects of PKA activation (i.e., decrease in myofilament Ca²⁺ sensitivity) (18, 23).

PKC phosphorylates also cMyBP-C but the functional significance of these phosphorylations in the control of cardiac function is unknown (22, 24). Interestingly, PKC leads to CaMKII activation upon Epac activation, suggesting that a linear cascade involving PLC, PKC, and CaMKII could be involved in Epac-dependent myofilament regulation (25) (Fig. S7). Alternatively, CaMKII could directly phosphorylate cTnI and cMyBP-C. Indeed, we found that inhibition of CaMKII activity blocked the effect of Epac on myofilament Ca²⁺ sensitivity and phosphorylation of sarcomeric proteins. The residual phosphorylation of cTnI and cMyBP-C could be because of PKC activation by Epac. Consistent with our study, cMyBP-C has also been reported to be phosphorylated by CaMKII (26–28), resulting in modulation of the rates of force development and relaxation (29). It is now known that cardiac contraction can be differentially regulated by a restricted

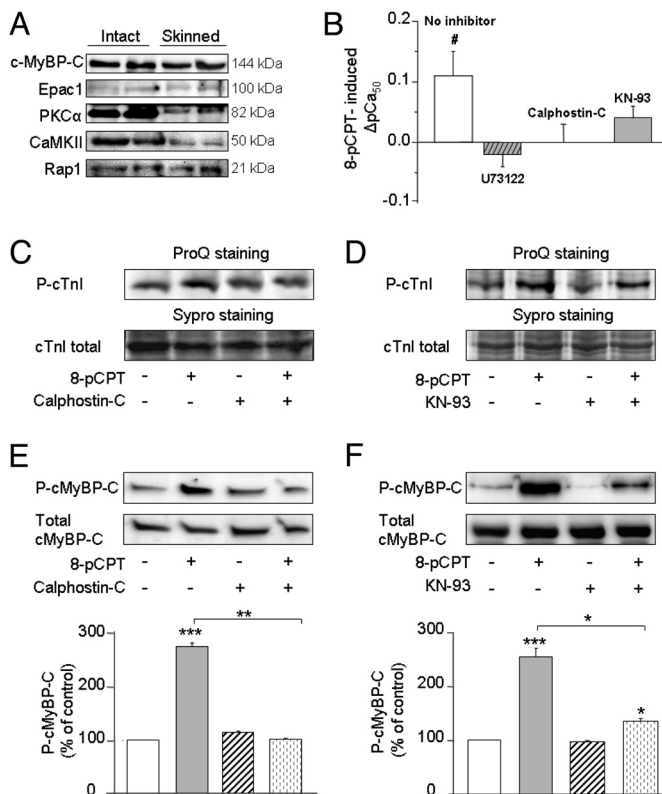


Fig. 5. Epac signaling pathways involved in myofilament protein regulation. (A) Expression analysis of Epac and its potential downstream effectors in nonpermeabilized (intact) and permeabilized cardiac myocytes. The indicated proteins were revealed by Western blotting as described in *SI Materials and Methods*. (B) Shift of myofilament Ca^{2+} sensitivity of activation ($\Delta p\text{Ca}_{50}$) induced by 8-pCPT in permeabilized cardiomyocytes preincubated with a PLC inhibitor (U73122, 5 μM), a PKC inhibitor (Calphostin C, 0.5 μM) or a CaMKII inhibitor (KN-93, 2 μM). Average values were expressed as the difference in $p\text{Ca}_{50}$ between nonstimulated (no inhibitor) and 8-pCPT-treated cells ($n = 8\text{--}13$ cells per condition). (C and E) Effect of a PKC inhibitor, calphostin-C (0.5 μM), and (D and F) effect of a CaMKII inhibitor, KN-93 (0.5 μM), on P-cTnI and P-cMyBP-C in isolated cardiomyocytes. In C and D phosphorylation of the 23-kDa band corresponding to P-cTnI was revealed by ProQ Diamond phosphoprotein gel staining, followed by total protein staining with Sypro Ruby to confirm equal loading. Representative gels are shown of 4 independent experiments. (E and F) Western blots were performed to probe for P-cMyBP-C and total cMyBP-C expression. Lower, immunoblots were quantified and data normalized to total cMyBP-C expression. Results are means \pm SEM from 5 (E) and 4 (F) independent experiments. *, $P < 0.05$; ***, $P < 0.001$ compared with control and indicated values.

number of sarcomeric proteins (22). Indeed, phosphorylation of cTnI and cMyBP-C can exert opposite regulatory effects depending on the type of kinase involved and on the site of phosphorylation. Our data suggest that the functional effect of Epac on myofilaments results from phosphorylations of both cTnI and cMyBP-C following dual activation of PKC and CaMKII (Fig. 5). This particular pathway may explain the discrepancies between the myofilament Ca^{2+} sensitization observed in the present study and the myofilament Ca^{2+} desensitization observed after specific PKC stimulation in other studies (18, 23). Interestingly, PKC isoforms differentially regulate sarcomeric protein phosphorylation such as cTnI, MLC-2, and TnT (30, 31). In our study, we did not find any effect of Epac activation on the phosphorylation status of myosin light chain 2 (Fig. S8). Consistent with our data on PKC-dependent myofilament sensitization upon Epac activation, the PKC-dependent pathway involving the phosphoryla-

tion of cTnI has been previously shown to increase myofilament Ca^{2+} sensitivity (31, 32). At present, we still do not know which PKC isoform(s) is (are) specifically involved in the regulation of myofilament protein phosphorylation induced by Epac. Further studies will be needed to identify the specific Epac-dependent phosphorylation sites in cTnI and cMyBP-C.

An important question concerns the physiological or pathophysiological relevance of Epac effects on the phosphorylation of myofilament protein. We show here that ISO increases cMyBP-C phosphorylation in a PKA-independent fashion, suggesting that β -AR may influence sarcomeric phosphorylation via Epac. It is therefore crucial to understand the context in which Epac is regulated by β -AR and the associated functional effects. Epac activity depends on the level of cAMP, which is in turn regulated by adenylate cyclases and phosphodiesterases (PDE). Some PDE inhibitors, such as adibendan and saterinone referenced as Ca^{2+} sensitizers (33), behave like Epac. Because PDEs are key enzymes for the regulation of cAMP concentration and diffusion in cardiac cells (34), one can speculate that PDE inhibitors may influence the Epac signaling pathway to account for their Ca^{2+} sensitizing effects.

Chronic stimulation of β -AR causes hypertrophy in cardiac myocytes (35). Our previous work indicated that Epac contributes to the hypertrophic effect of β -AR in a CaMKII-dependent, but PKA-independent, fashion (19). Consistent with its role in cardiac remodeling, Epac is increased in different animal models of myocardial hypertrophy and upregulates markers of cardiac hypertrophy (12, 19, 36). Altogether these findings, combined with the observation that myofilament properties are altered in cardiac hypertrophy and heart failure, suggest that Epac may play a role in the changes of sarcomeric proteins observed in these pathologies (3).

In conclusion, our data show that the cAMP-binding protein Epac has an opposite effect from PKA on myofilament Ca^{2+} sensitivity. We demonstrate that Epac is a mediator of sarcomeric proteins' phosphorylation and may contribute to the regulation of myofilament function. Our data suggest that altered Epac activity in disease may impact on contractile function.

Materials and Methods

Myocyte Preparation. 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. Male Wistar rats (250–300 g) were anesthetized by sodium pentobarbital i.p. injection (2 g/kg). Cardiac ventricular myocytes were isolated by standard enzymatic methods as previously described (37). Briefly, the heart was excised and perfused retrogradely for 5 min at 37 $^{\circ}\text{C}$ with a Ca^{2+} -free HEPES-buffered solution (117 mM NaCl, 5.7 mM KCl, 4.4 mM NaHCO_3 , 1.5 mM KH_2PO_4 , 1.7 mM MgCl_2 , 21 mM HEPES, 11 mM glucose, 20 mM taurine) adjusted to pH 7.2 with NaOH and bubbled with 100% O_2 . The heart was then perfused with an enzyme-containing solution for 20–30 min (1.3 $\text{mg}\cdot\text{mL}^{-1}$ collagenase type IV (Worthington). Cells were then filtered and washed several times in the HEPES-buffered solution containing 0.3 mM Ca^{2+} . Finally, myocytes were kept in HEPES-buffered solution containing 1 mM Ca^{2+} and 0.5% BSA. The specific relationship between the amount of Ca^{2+} and the force developed by myofilaments was studied in myocytes permeabilized with 0.3% Triton X-100 in relaxing solution, resulting in a full permeabilization of sarcolemma, SR, nuclear, and mitochondrial membranes. Myofilaments are activated by perfusing the cell with an internal solution containing increasing amounts of Ca^{2+} . With permeabilized myocytes it is thus possible to measure precisely the relationship between force developed by the myofilaments and the exact amount of Ca^{2+} . Permeabilized cells were incubated with either 8-pCPT (1 μM , 10 min) or 6-Bnz-cAMP (200 μM , 60 min) in relaxing solution at room temperature (22 $^{\circ}\text{C}$) to activate Epac and PKA, respectively. We also activated the PKA pathway, using the recombinant catalytic subunit of PKA. For that, permeabilized cells were preincubated for 50 min at room temperature with 200 UI of recombinant catalytic subunits of PKA per milliliter of relaxing solution.

For a description of other methods, see *SI Materials and Methods*.

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