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EPAC1 and 2 inhibit K⁺ currents via PLC/PKC and NOS/PKG pathways in rat ventricular cardiomyocytes

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Keywords:

8-CPTAM; EPAC; action potential; cardiac myocyte; potassium current.

Abstract

The exchange protein directly activated by cAMP (EPAC) has been implicated in cardiac proarrhythmic signaling pathways including spontaneous diastolic Ca^{2+} leak from sarcoplasmic reticulum and increased action potential duration (APD) in isolated ventricular cardiomyocytes. The action potential (AP) lengthening following acute EPAC activation is mainly due to a decrease of repolarizing steady-state K^+ current (IK_{SS}) but the mechanisms involved remain unknown. This study aimed to assess the role of EPAC1 and EPAC2 in the decrease of IK_{SS} and to investigate the underlying signaling pathways. AP and K^+ currents were recorded with the whole cell configuration of the patch-clamp technique in freshly isolated rat ventricular myocytes. EPAC1 and EPAC2 were pharmacologically activated with 8-(4-chlorophenylthio)-2'-*O*-methyl-cAMP acetoxymethyl ester (8-CPTAM, 10 $\mu\text{mol/L}$) and inhibited with R-Ce3F4 and ESI-05, respectively. Inhibition of EPAC1 and EPAC2 significantly decreased the effect of 8-CPTAM on APD and IK_{SS} showing that both EPAC isoforms are involved in these effects. Unexpectedly, calmodulin-dependent protein kinase II (CaMKII) inhibition by AIP or KN-93, and Ca^{2+} chelation by intracellular BAPTA, did not impact the response to 8-CPTAM. However, inhibition of PLC/PKC and nitric oxide synthase (NOS)/PKG pathways partially prevents the 8-CPTAM-dependent decrease of IK_{SS} . Finally, the cumulative inhibition of PKC and PKG blocked the 8-CPTAM effect, suggesting that these two actors work along parallel pathways to regulate IK_{SS} upon EPAC activation. On the basis of such findings, we propose that EPAC1 and EPAC2 are involved in APD lengthening by inhibiting a K^+ current via both PLC/PKC and NOS/PKG pathways. This may have pathological implications since EPAC is upregulated in diseases such as cardiac hypertrophy.

NEW & NOTEWORTHY

Exchange protein directly activated by cAMP (EPAC) proteins modulate ventricular electrophysiology at the cellular level. Both EPAC1 and EPAC2 isoforms participate in this effect. Mechanistically, PLC/PKC and nitric oxide synthase (NO)/PKG pathways are involved in regulating K^+ repolarizing current whereas the well-known downstream effector of EPAC, calmodulin-dependent protein kinase II (CaMKII), does not participate. This may have pathological implications since EPAC is upregulated in diseases such as cardiac hypertrophy. Thus, EPAC inhibition may be a new approach to prevent arrhythmias under pathological conditions.

INTRODUCTION

Cyclic adenosine 3',5'-monophosphate (cAMP) is a universal intracellular second messenger that regulates numerous physiological and pathological processes. In heart, an increase in cAMP, resulting from sympathetic stimulation of β -adrenergic receptors, controls cardiac function such as heart rate and excitation-contraction (EC) coupling in both healthy and pathological conditions. cAMP downstream effects in ventricle were first attributed to cAMP-dependent protein kinase (PKA) which, subsequently, phosphorylates key proteins in the EC coupling process (1). However, this paradigm was challenged with the discovery of exchange proteins directly activated by cAMP (EPAC), adding a new level of complexity to the cAMP-mediated signaling cascade. Both isoforms of EPAC proteins (named EPAC1 and EPAC2 respectively) are guanine nucleotide exchange factors (GEF) for small GTPases Rap1 and Rap2 and their effects are PKA independent (2, 3). In heart, both EPACs are expressed with a predominance for EPAC1 (2, 3).

At the ventricular level, EPAC was implicated in myocyte Ca^{2+} homeostasis perturbations leading to cardiomyocyte hypertrophy (4–6) and proarrhythmic effects (7, 8). Direct pharmacological activation of EPAC with the cAMP analog 8-(4-chlorophenylthio)-20-O-methyl-cAMP (8-CPT) promotes ventricular arrhythmogenesis in isolated perfused mouse hearts (7). These arrhythmias were subsequently attributed to an increase of diastolic Ca^{2+} leak from the sarcoplasmic reticulum (SR) through ryanodine receptors (RyRs) (8) probably due to an increase in diastolic Ca^{2+} spark occurrence (9). Diastolic SR Ca^{2+} leak is recognized as a trigger for the development of delayed afterdepolarizations (DAD), a major source of ectopic activity (10). In addition, a decrease in conduction velocity has been reported following pharmacological EPAC activation in both whole heart (8) and atria (11) that may result from a decrease in the fast component of the Na^{+} current (I_{Na}) (12). In rat ventricular cardiomyocytes, acute EPAC activation lengthens action potential duration (APD) by decreasing the sustained (i.e., non-inactivating) component of K^{+} current (13), a process which is correlated with the genesis of arrhythmia by predisposing cardiomyocytes to early afterdepolarization (EAD) and dispersion of repolarization (14). Moreover, an EPAC-dependent increase in the late component of the fast Na^{+} current ($I_{\text{Na, L}}$) may be involved in reshaping cardiac action potential (AP) and its arrhythmogenic potential (15). AP prolongation was also reported during chronic activation of EPAC in guinea pig ventricular cardiomyocytes by decreasing the expression level of MinK, a subunit of the slow delayed rectifier potassium (IK_s) voltage-gated channel (KCN) (16). Altogether, these studies strongly suggest the proarrhythmogenic potential of EPAC activation in the heart.

Mechanistically, several studies in isolated cardiac myocytes have shown that stimulation of EPAC by 8-CPT increased the activity of calmodulin-dependent protein kinase II (CaMKII). The downstream phosphorylation of two sarcoplasmic reticulum (SR) targets, the Ca^{2+} release channel (RyR2) and phospholamban, the Ca^{2+} pump (SERCA2a) regulator, has also been shown (9, 17–22). Moreover, at the level of the myofibrils, 8-CPT enhanced the CaMKII-dependent phosphorylation of myosin-binding protein C and troponin I (23). Even though CaMKII appears as a clear downstream signal of EPAC, other signaling pathways have been proposed. The EPAC-mediated effects have been shown to require the presence of ϵ isoform of phospholipase C ($\text{PLC}\epsilon$) (18, 19) and the resultant increase in cytosolic Ca^{2+} triggered by inositol triphosphate (IP_3) as well as the diacylglycerol (DAG)-activated $\text{PKC}\epsilon$, have been implicated in the stimulation of CaMKII under different experimental conditions (9, 18, 19). In addition, nitric oxide synthase (NOS) and phosphoinositide 3-kinase-dependent activation

of CaMKII have been reported during EPAC stimulation (15, 20, 21). In cardiomyocytes, it is well demonstrated that EPAC modulates Ca²⁺ homeostasis and CaMKII inhibition prevents 8-CPT-induced SR Ca²⁺ leak (9, 22) and the occurrence of 8-CPT-induced ectopic Ca²⁺ transients (7).

In contrast, the modulation of ion channels by EPAC remains poorly investigated. In cardiomyocytes, acute activation of EPAC by 8-CPT leads to a decrease in sustained K_p current but the mechanism remains unknown (13). Na_p currents are modulated but via the effect of EPAC on calcium homeostasis, either directly [increase [Ca²⁺] (7)] or via CaMKII (15). In other cell types, EPAC activation can modulate ion channel activity but again the underlying mechanisms remain unclear. In endothelial cells, EPAC activates calcium-sensitive K_p channels (24). In sensory neurons, the EPAC-selective cAMP analog 8-pCPT sensitizes mechanically evoked currents (Piezo2) (25). Kv7 currents are increased with direct EPAC stimulation in smooth muscle cells isolated from arteries (26). In all these studies it is unclear whether there is a direct or indirect effect on the channel. In addition, non-canonical EPAC signaling has been shown to affect KATP channel open probability in pancreatic b cells (27) and GABAAR dynamics in neurons (28). Thus, although it is clear that in cardiomyocytes EPAC modulates Ca²⁺ homeostasis via CaMKII and also impacts other pathways important for cell function, further studies are needed to determine the signaling pathway specific to cardiac ion channels, especially sustained K_p currents (13) due to their role in arrhythmogenesis.

The main objective of the present study was to determine what role EPAC1 and EPAC2 might play in K_p current modulation in cardiomyocytes. To achieve this goal, we dissected the signaling pathway linking EPAC activation to AP prolongation. We used a pharmacological approach in freshly dissociated ventricular cardiomyocytes from rat. We show that EPAC1 and EPAC2 are both involved in APD prolongation via interaction with the sustained K_p-current. Unexpectedly, we show the CaMKII signaling pathway is not involved, rather, PLC/PKC and NOS/PKG signaling pathways underlie the modulation of IK.

MATERIALS AND METHODS

Animal Ethics and Animal Care

All experiments were approved and conducted in accordance with the recommendations of the local ethic committee, the EU directive 2010/63/EU and the ARRIVE guidelines for reporting the use of animals in scientific experiments. For this study, male Wistar rats (225–300 g) were used. Rats were housed on a 12:12 h light-dark cycle in a temperature regulated room (23 ± 2 °C) with ad libitum access to food and water.

Isolation of Rat Ventricular Myocytes

Cardiac ventricular myocytes were isolated as previously described (13). Briefly, rats were anesthetized by inhalation of isoflurane (5% a mix of 30% O₂ and 70% N₂O) and rapidly killed by cervical dislocation. Then, the heart was quickly excised and mounted in a Langendorff apparatus. The heart was primarily perfused with a modified Tyrode solution containing (in mmol/L): NaCl 130, KCl 5.4, MgCl₂ 1.4, NaH₂PO₄ 0.4, Glucose 10, Taurine 20, Creatine 10, HEPES 4.2, and CaCl₂ 1; pH 7.4 with NaOH to clear vessels of their blood. Once coronaries had cleared, perfusion solution was switched to a Ca²⁺-free Tyrode solution (CaCl₂ was replaced by 0.1 mmol/L EGTA) to predigest the heart for 5 min. The predigest

was then followed by an enzymatic Tyrode solution containing type 1 collagenase (150 U/mL—Worthington Chemicals, Laekwood, NJ) and type XIV protease (4 mg—Sigma Aldrich, L'Isle d'Abeau Chesnes, France) from 10 to 15 min, according to the digestion status of the tissue.

Ventricles were then excised from the heart, minced, and placed in an oxygenated enzymatic solution supplemented by 0.1% BSA, and gently shaken at 37°C. Ventricular cells were filtered at 5-min intervals, centrifuged at 24 g, and resuspended in a low Ca²⁺ (0.5 mmol/L) isolation Tyrode solution. Ca²⁺ was then gradually reintroduced until reaching the final Ca²⁺ concentration (1 mmol/L). Only ventricular striated cells were used for experiments. Experiments were performed at room temperature (20–25°C) during the 8 h following cell isolation.

Patch-Clamp Experiments

Cardiac myocytes were studied in Petri dishes on the stage of an inverted microscope (Nikon TE200-S, Japan). Action potentials and potassium currents were recorded with the whole cell configuration of the patch clamp technique. An Axopatch 200B amplifier (Molecular Devices) connected to a digidata 1322 A/D (Molecular Devices) was used and data were analyzed using pClamp 9 (Molecular Devices). Signals were digitized at a frequency of 10 kHz and filtered at 2 kHz using an 8-pole Bessel low-pass filter. Resistance of the patch pipettes was from 0.8 to 1.7 MΩ when filled with the intrapipette solution.

Action Potentials were elicited by a 2.5-ms supra-threshold square current stimulation and recorded at a frequency of 0.1 Hz. The extracellular medium was the modified Tyrode solution used for cell isolation. Intrapipette solution was composed of (in mmol/L): KCl 130, NaCl 10, MgCl₂ 0.5, HEPES 10 and EGTA 0.5, pH 7.2 with KOH.

Potassium currents were recorded in the same Tyrode solution as the APs, except that 1 mmol/L of CdCl₂ was delivered by perfusion to block the fast Na⁺, Ca²⁺, and Na⁺-Ca²⁺ exchanger currents. Intrapipette medium for potassium currents recording was composed of (in mmol/L): KCl 140, MgCl₂ 0.5, HEPES 10, EGTA 5, Mg-ATP 5, and GTP Tris salt 0.4, pH 7.2 with KOH. To elicit K⁺-currents, cells were maintained at a holding level of -80 mV and a 300-ms square test pulse to +30 mV was applied at a frequency of 0.1 Hz. To ensure full inactivation of sodium currents during the test pulse, a 50-ms pre-pulse to -40 mV was used.

To record the steady-state component IK_{SS}, a 300-ms test pulse was applied to +30 mV at a frequency of 0.1 Hz with a holding level of -20 mV (to inactivate the transient outward component I_{to} and I_K).

Data Analysis

AP amplitude was measured as the difference between the peak of overshoot and the resting membrane potential. The depolarization velocity (dV/dt_{max}) was calculated by Clampfit software (the pClamp 9 analysis module, Molecular Devices) by differentiation of the AP maximal upstroke. APD was measured at three percentages of repolarization (20%: APD₂₀; 50%: APD₅₀ and 90%: APD₉₀, respectively) as the duration from the peak of overshoot to the three different percentages of repolarization. For K⁺ current analysis, the non-inactivating sustained component (I_{Ksus}) was measured as the steady current remaining at the end of the 300-ms test pulse from a holding potential of -80 mV. The transient inactivating component

Ito was measured as the difference between the outward peak component and IKsus. The steady-state component IKSS was measured as the steady current at the end of the 300-ms test pulse from a holding potential of -20 mV. Cells showing more >10% change in Ihold (i.e., current at -80 mV) during experiments were discarded from analysis.

Chemicals

The description and use of the different pharmacological compounds are presented in Supplemental Table S1. To measure the effect of EPAC activation on AP or currents, 8-(4-Chlorophenylthio)-20-O-methyl-cAMP acetoxymethyl ester (8-CPTAM) was provided by perfusion at least 15 min after reaching the ruptured configuration. To ensure a good delivery of the different inhibitors into cells, they were systematically preincubated during at least 1 h before patch-clamping and provided by perfusion system without 8-CPTAM during at least 15 min after reaching the ruptured configuration. Then, 8-CPTAM p inhibitor solution was provided to the cell by perfusion. In addition, inhibitors were added in the intrapipette medium when possible (Supplemental Table S1).

Statistics

Data are presented as means \pm SE. N and n are the number of animals and the number of cells, respectively. Statistical analysis and graphical representation were performed using Sigma plot 11 and Graphpad Prism 8 software, respectively. A paired t test was used to compare data from control and treated cells after validation of normal distribution by a Shapiro–Wilk test and equal variance by an exact Fisher test. Unpaired t test and one-way ANOVA followed by Holm– Sidak post hoc test were used for comparison between two groups or several groups, respectively. When normality and/ or equal variance were not validated, nonparametric tests (Mann–Whitney, Wilcoxon, or Kruskal–Wallis test with Dunn’s post hoc) were used as appropriate. $P < 0.05$ was considered as significant.

RESULTS

Both EPAC1 and EPAC2 Are Involved in APD Lengthening

We have previously reported an increase of APD under acute application of EPAC (13). Here, we confirm that 8- CPTAM at 10 μ mol/L significantly increased APD (APD20 APD50, and APD90 respectively, Fig. 1A), whereas other AP parameters such as resting membrane potential, AP amplitude, dV/dtmax were not changed (Supplemental Fig. S1). APD20, APD50, and APD90 were lengthened by $11.86 \pm 3.91\%$, $32.83 \pm 8.66\%$, and $37.00 \pm 7.41\%$ respectively ($P = 0.011$ for APD20, $P < 0.001$ for APD50 and APD90).

To investigate which EPAC isoform is responsible for APD modulation, we pretreated cells with selective inhibitors of EPAC1 (R-Ce3F4, 10 μ mol/L) or EPAC2 (ESI-05, 10 μ mol/L). Under basal conditions, these treatments did not affect any AP parameters (Supplemental Fig. S2).

When 8-CPTAM was perfused in combination with ESI-05, only APD90 remained increased but to a significant lower value compared with the 8-CPTAM effect alone ($10.33 \pm 4.78\%$

vs. $37 \pm 7.41\%$, $P = 0.02$; [Fig. 1, B and E](#)). Thus, EPAC2 is involved in the EPAC-dependent APD lengthening.

In R-Ce3F4 pretreated cells, perfusion of 8-CPTAM significantly lengthened APD50 and APD90 (by $19.80 \pm 7.37\%$, $P = 0.004$ and $10.45 \pm 4.01\%$, $P = 0.048$ respectively; [Fig. 1C](#)). However, and similar to ESI-05 pretreated cells, the AP lengthening was significantly less than that observed in the control condition ($P = 0.015$, [Fig. 1E](#)). These data show that both EPAC1 and EPAC2 are important in APD modulation, but EPAC1 and EPAC2 activities are redundant so that activity of either isoform can fulfill this requirement in the absence of the other. To further test this hypothesis, we performed pharmacological inhibitions of both EPAC1 and EPAC2 (combined treatment with R-Ce3F4 and ESI-05). We observed that 8-CPTAM did not increase APD ([Fig. 1, D and E](#)).

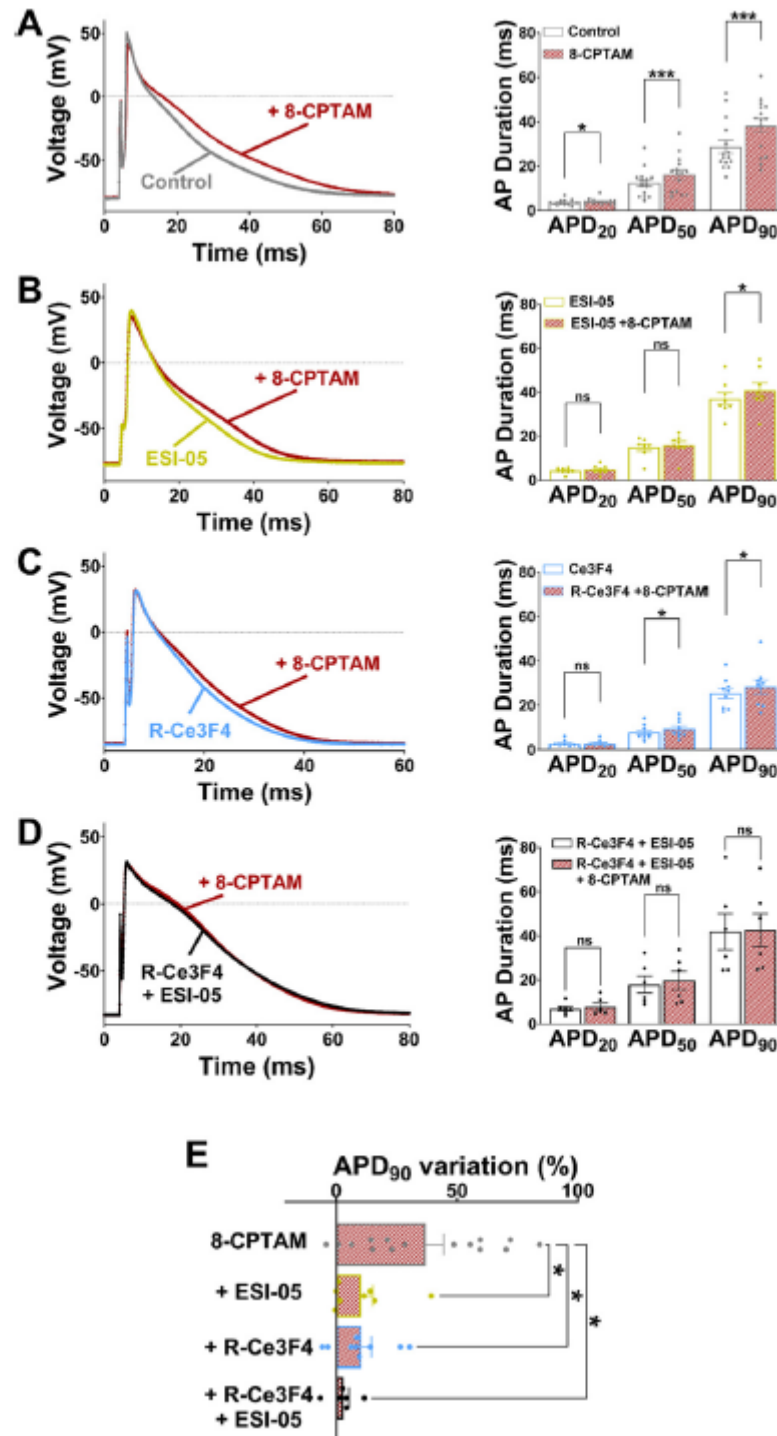


Figure 1. Both exchange proteins directly activated by cAMP (EPAC)1 and EPAC2 are involved in 8-(4-chlorophenylthio)-20-O-methyl-cAMP acetoxymethyl ester (8-CPTAM)-dependent action potential (AP) lengthening. A: effect of 8-CPTAM on action potential duration (APD) (N = 7; n = 15). B and C: reduction of the 8-CPTAM effect by selective inhibition of EPAC2 (ESI-05, N = 3, n = 8) and EPAC1 (R-Ce3F4, N = 3, n = 9), respectively. D: combined pretreatment with ESI-05 and R-Ce3F4 abolished 8-CPTAM effect (N = 3, n = 6). E: comparison of the 8-CPTAM relative APD lengthening in nontreated, ESI-05 pre-treated, R-Ce3F4 pretreated, and combined pretreated cardiomyocytes. Data are means \pm SE. Statistical differences were determined by paired t test or Wilcoxon test when normality was not confirmed. Unpaired t tests or Mann–Whitney test when normality was not confirmed were used in (E). *P < 0.05; ***P < 0.001.

Both EPAC1 and EPAC2 Are Involved in Repolarizing K_p Current Decrease

Our previous work has shown that EPAC-dependent AP prolongation is due to a decrease in the sustained component of K_p current (13). To determine the respective role of EPAC1 and EPAC2 on K_p current modulation, we used the voltage clamp mode of the patch-clamp technique on cardiomyocytes. Transient outward current (I_{to}) was not changed by acute application of 10 μmol/L 8-CPTAM (8.25 ± 1.38 pA/pF vs. 7.83 ± 1.23 pA/pF in control and 8-CPTAM respectively, $P = 0.489$, Fig. 2B), whereas the non-inactivating component of K_p current (I_{Ksus}) was reduced by $36.3 \pm 1.81\%$ (5.7 ± 0.42 pA/pF vs. 3.63 ± 0.33 pA/pF, $P < 0.001$, Fig. 2B). When we specifically recorded the steady-state component (IKSS) of I_{Ksus} from a holding potential at -20 mV, IKSS measured at -30 mV was decreased by $39.26 \pm 5.98\%$ following 8-CPTAM application (2.1 ± 0.28 pA/pF vs. 1.17 ± 0.09 pA/pF, $P = 0.002$, Fig. 2B). These results are consistent with our previous data (13).

In cardiomyocytes pretreated with 25 μmol/L of R-Ce3F4, addition of 8-CPTAM significantly decreased both I_{Ksus} and IKSS by $20.99 \pm 3.42\%$ and $23.69 \pm 5.27\%$ ($P < 0.01$ for each, Fig. 3A), respectively. The 8-CPTAM effect was significantly lower in the presence of R-Ce3F4 for I_{Ksus} ($P < 0.01$) and tended to decrease IKSS ($P = 0.08$, Fig. 3D) suggesting an EPAC1 contribution in the decrease of K_p current. When 25 μmol/L of ESI-05 was used as pretreatment to inhibit EPAC2, I_{Ksus} and IKSS remained decreased by $21.04 \pm 3.79\%$ ($P < 0.05$) and $19.55 \pm 6.03\%$ ($P < 0.05$, Fig. 3B). Nevertheless, ESI-05 significantly limited the 8-CPTAM effect on I_{Ksus} and IKSS ($P < 0.01$ and $P < 0.05$, respectively, Fig. 3D). Taken together these data clearly show that both EPAC isoforms are involved in K_p currents modulation. Combined treatment with R-Ce3F4 and ESI-05 fully inhibited the effect of 8-CPTAM on IKSS (1.58 ± 0.13 vs. 1.53 ± 0.13 pA/pF, $P = 0.233$) and partially (by 70%) on I_{Ksus} (5.18 ± 0.56 vs. 4.67 ± 0.55 pA/pF, $P < 0.001$) confirming the contribution of the two EPAC isoforms and the absence of off-target effects of 8-CPTAM application (Fig. 3, C and D).

Taken together, these results show that EPAC1 and EPAC2 are involved in the decrease of repolarizing K_p current. We next thought to explore the signaling pathway(s) responsible for this.

Effect of EPAC Activation on K_p Current Is Independent of CaMKII

Because RyR phosphorylation by CaMKII at its Ser2815 site is increased upon EPAC activation (17, 18) and CaMKII is considered a downstream effector of EPAC, we first investigated whether the effect of 8-CPTAM was modulated by CaMKII inhibitors AIP or KN-93. Unexpectedly, both drugs failed to inhibit the 8-CPTAM effect on I_{Ksus} and IKSS. When 8-CPTAM was applied in the presence of AIP (20 μmol/L in pipette) pretreated cells, the inhibition of I_{Ksus} and IKSS was $33.94 \pm 2.4\%$ and $33.8 \pm 8.12\%$, respectively (Fig. 4, A and C). This result was reproduced when cells were in the presence of 2 μmol/L of KN-93 ($-38.09 \pm 4.05\%$ for I_{Ksus} and $-33.58 \pm 8.35\%$ for IKSS in the presence of 8-CPTAM, Fig. 4, B and C). These data are strong evidence that CaMKII is not involved in the EPAC-dependent modulation of K_p current in cardiomyocytes.

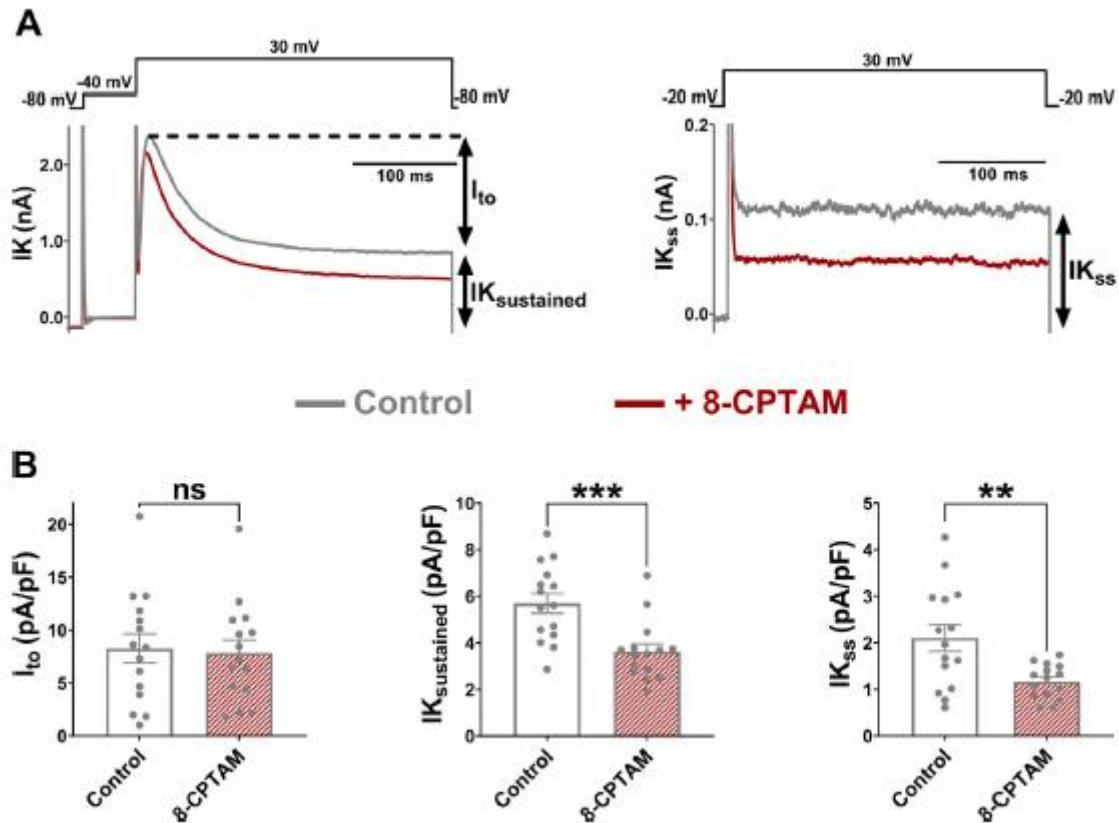


Figure 2. Exchange proteins directly activated by cAMP (EPAC) pharmacological activation downregulates sustained components of K β currents. A: representative traces illustrating the 8-(4-chlorophenylthio)- 20-O-methyl-cAMP acetoxyethyl ester (8-CPTAM)-dependent decrease of sustained component of potassium current (IK_{sus}) and steady-state potassium current (IK_{ss}). B: means \pm SE of transient outward potassium current (I_{to}), IK_{sus} and IK_{ss} currents measured at +30 mV in control and 8-CPTAM treated conditions (N = 5, n = 15). Paired t tests were used for statistical comparisons of IK_{sus} and IK_{ss}, and Wilcoxon test was used to compare I_{to}. **P < 0.01 and ***P < 0.001.

PLC and PKC Are Involved in a Calcium-Independent EPAC-Induced Modulation of K1 Current

Since PLC and PKC have been implicated in EPAC-mediated signaling, we next explored the putative involvement of both enzymes in the effect of 8-CPTAM on K β current using inhibitors U73122 and calphostin C for PLC and PKC, respectively. Both inhibitors were able to block partially the effect of 8-CPTAM on IK_{ss} and IK_{sus} (Fig. 5, A and D). In the presence of U73122 (5 μ mol/L), application of 8-CPTAM reduced both IK_{ss} and IK_{sus} by $9.19 \pm 4.76\%$ and $21.91 \pm 2.01\%$, respectively (P < 0.001 vs. 8-CPTAM alone for both currents). Alternatively, U73343, the inactive derivative of U73122 (5 μ mol/L) did not change the 8-CPTAM-mediated effect (Fig. 5, B and D). Because U73122 is not a specific PLC inhibitor (29–35), we further investigated the involvement of PLC/PKC pathway using the specific PKC inhibitor calphostin C. When calphostin C (2 μ mol/L) was provided to cells before and during 8-CPTAM application, the effect of the latter was inhibited for IK_{ss} ($-18.29 \pm 4.86\%$, P = 0.015 vs. 8-CPTAM alone) and for IK_{sus} ($-21.89 \pm 2.73\%$, P < 0.001 vs. 8-CPTAM alone) (Fig. 5, C and D). These data provide evidence for PLC and PKC as downstream effectors of EPAC in the regulation of non-inactivating K β currents.

In addition of activating PKC via DAG, PLC is known to produce IP3 which is likely to trigger a Ca^{2+} release from SR via inositol triphosphate receptor (IP3R) opening following EPAC activation (5, 6). Moreover, EPAC activation increases diastolic Ca^{2+} leak from the SR by RyR (9, 22). To determine whether modification in $[Ca^{2+}]_i$ also plays a role in the modulation of K^+ currents induced by EPAC activation, we used the fast Ca^{2+} chelator BAPTA (10 mmol/L) instead of EGTA in the patch pipette. Interestingly, effect of 8-CPTAM was not changed in the presence of BAPTA for both IK_{sus} and IK_{SS} (Supplemental Fig. S3). The inability of BAPTA to affect the 8-CPTAM effect on K^+ channels activity strongly suggests that EPAC-mediated channel inhibition does not relies upon an increase in subsarcolemmal Ca^{2+} .

NOS and PKG Contributes to the Modulation of K1 Current by EPAC

Since an EPAC-NOS pathway has been described in cardiac myocyte (21), the putative involvement of NO-synthase in the modulation of K^+ currents with 8-CPTAM was investigated with the NOS inhibitor L-NG-Nitroarginine Methyl Ester (L-NAME) [1 mmol/L in extracellular medium, (36–40)]. The effect of 8-CPTAM was reduced by L-NAME to -40% for IK_{sus} ($P < 0.001$) and 70% ($P = 0.004$) for IK_{SS} compared with 8-CPTAM alone (Fig. 6, A and C). Because we excluded the involvement of CaMKII (which can be activated by S-nitrosylation) we tested the participation of the classical NO/GMPc/PKG pathway using KT5823 (1 μ mol/L), a selective inhibitor of PKG. Interestingly, KT5823 decreased the effect of 8-CPTAM on IK_{sus} and IK_{SS} by - 31% ($P = 0.006$) and 58% ($P = 0.02$) respectively (Fig. 6, B and C).

EPAC Activates Two Independent Pathways to Regulate K^+ Current

Next, we tested the hypothesis that EPAC could stimulate two independent pathways to decrease IK_{sus} and IK_{SS} using a combination of calphostin C and KT5823. When these two inhibitors were applied before and during 8-CPTAM perfusion, the effect of the EPAC activator was less pronounced on IK_{sus} ($P < 0.01$ vs. Calphostin C and $P = 0.051$ vs. KT5823, Fig. 7A), but not on IK_{SS} compared with when they were used alone (Fig. 7B) suggesting a dual independent effect of PKC and PKG in the regulation of the non-inactivating component of K^+ current.

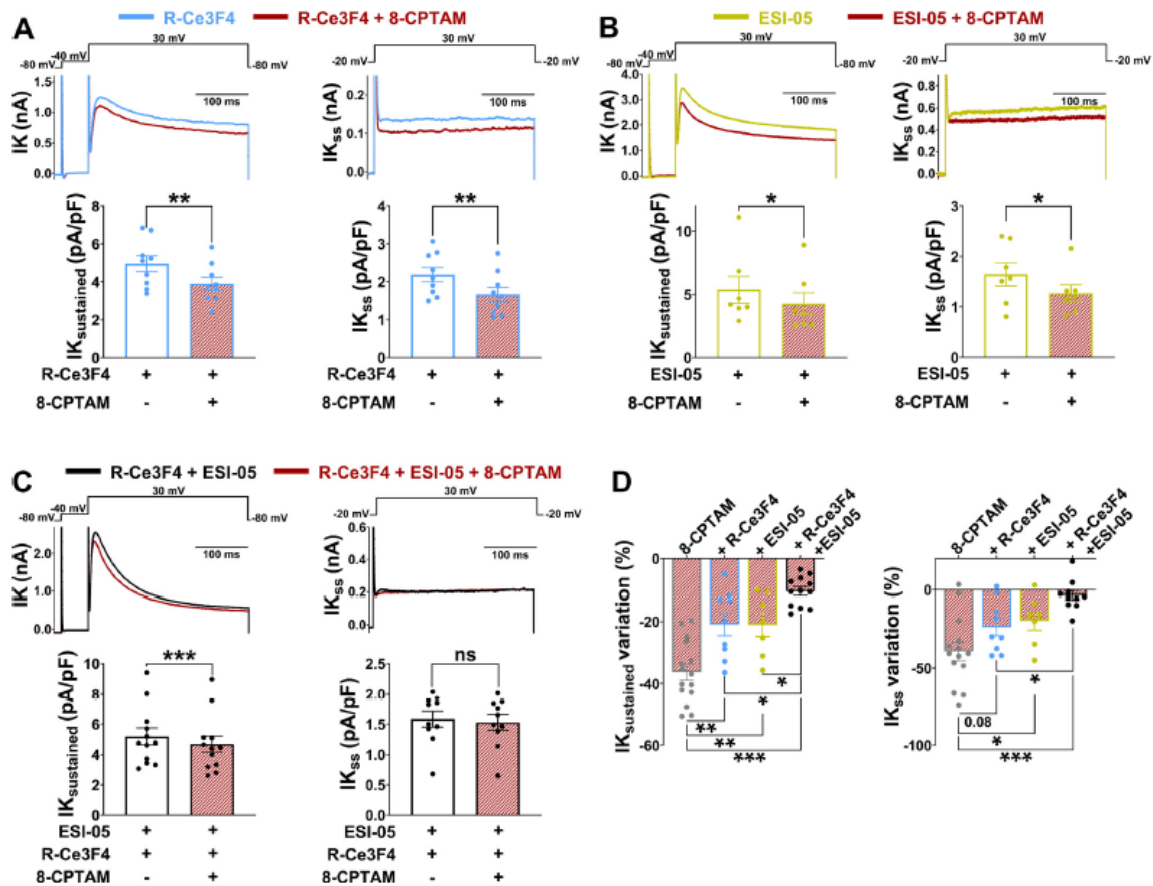


Figure 3. Both exchange proteins directly activated by cAMP (EPAC)1 and EPAC2 modulate sustained components of K_p currents. A–C: effect of 8-(4-chlorophenylthio)-20-O-methyl-cAMP acetoxymethyl ester (8-CPTAM) treatment on sustained component of potassium current (IK_{sus}) and steady-state potassium current (IK_{ss}) in cardiomyocytes pre-treated with 25 μmol/L of the EPAC1 inhibitor R-Ce3F4 (N = 3, n = 9), 25 μmol/L of the EPAC2 inhibitor ESI-05 (N = 2, n = 7) and combination of R-Ce3F4 and ESI-05 (N = 5, n = 12 for IK_{sus} and n = 10 for IK_{ss}), respectively. D: comparison of the 8-CPTAM-induced variation of IK_{sus} (left) and IK_{ss} (right) in non-pretreated cells and cells pretreated with each inhibitors or combination of both. Data are represented by means ± SE. Statistical analysis was performed with paired t tests in (A) and for the IK_{ss} data in (C), and with Wilcoxon test in (B) and for the IK_{sus} data in (C). One-way ANOVAs with Holm–Sidak post hoc were used in (D). *P < 0.05; **P < 0.01, and ***P < 0.001.

EPAC1 and EPAC2 Modulate K₁ Currents by a Common Signaling Pathway

With regards to the results described earlier, it was tempting to speculate that EPAC1 and EPAC2 could preferentially activate different pathways. We tested this hypothesis using combinations of inhibitors. R-Ce3F4 (25 μmol/L) or ESI-05 (25 μmol/L) was associated with Calphostin C (2 μmol/L) or KT5823 (1 μmol/L) and the effect of 8-CPTAM was assessed under these four conditions. Surprisingly, although the effect of 8-CPTAM remained limited by all the combinations (Supplemental Fig. S4, B and D), none of the four co-treatments displayed any supplemental benefit compared with the effect of each individual pretreatment on the sustained components IK_{sus} (Fig. 7C).

Co-treatment with every EPAC inhibitor and KT5823 entirely blocked the 8-CPTAM-dependent effect on IK_{ss} (P = 0.24 for R-Ce3F4 + KT5823 and P = 0.95 for ESI-05 +

KT5823; Supplemental Fig. 4, B and D), whereas other combinations failed to totally inhibit the effect of EPAC pharmacological activation. Taken together, these results support the idea of a common signaling pathway for EPAC1- and EPAC2-dependent modulation of K_p currents. In addition, it shows the involvement of a PKG-dependent pathway on IKSS modulation.

DISCUSSION

The aim of our study was to decipher the signaling pathways downstream of EPAC that modulate K_p currents in ventricular cardiomyocytes. The major findings are as follows: 1) EPAC1 and EPAC2 are involved in AP lengthening by inhibiting one component of the repolarizing K_p sustained current. 2) This effect does not depend on Ca_2p and CaMKII. 3) Two independent PLC/PKC and NO/PKG pathways act together in modulating K_p sustained current. 4) EPAC1 and EPAC2 do not appear to preferentially activate one pathway over another. We have summarized all these findings in Fig. 7E.

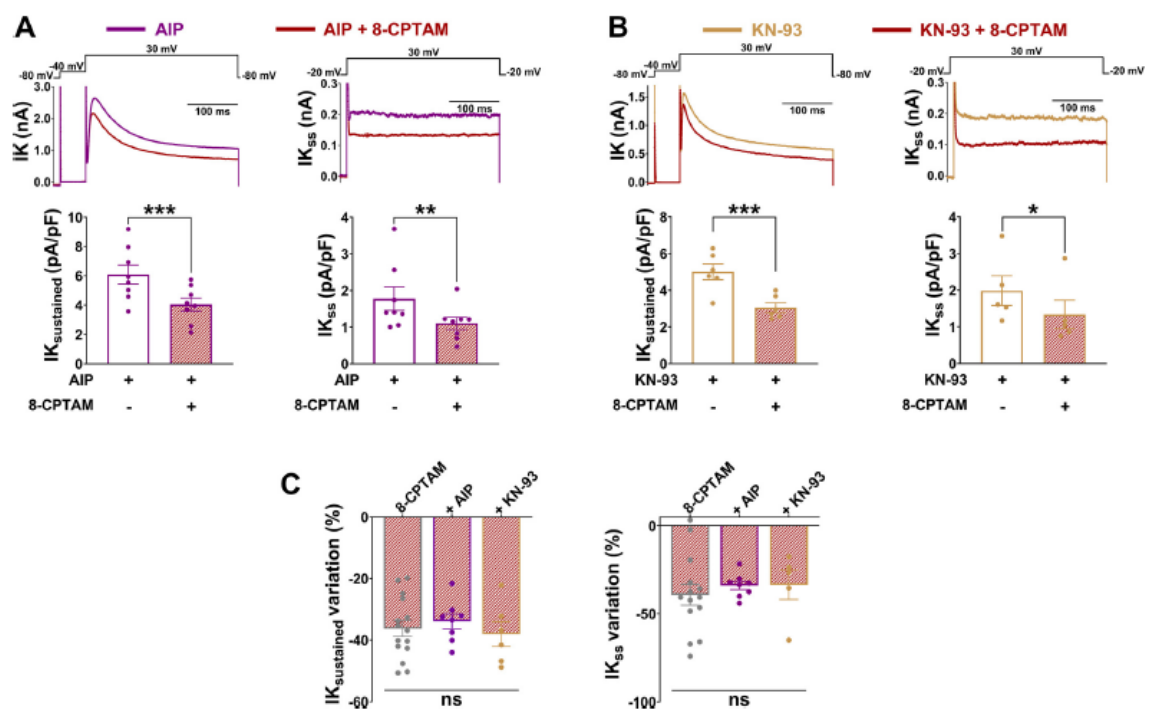


Figure 4. The effect of exchange proteins directly activated by cAMP (EPAC) on K_p currents is calmodulin-dependent protein kinase II (CaMKII) independent. A: the CaMKII inhibitor AIP (20 μ mol/L provided by the intrapipette medium) failed to prevent the 8-(4-chlorophenylthio)-20-O-methyl-cAMP acetoxymethyl ester (8-CPTAM)-induced variation of sustained component of potassium current (IK_{sus}) and steady-state potassium current (IK_{ss}) (N = 3, n = 8). B: EPAC-dependent inhibition of K^+ currents is preserved in KN-93 pretreated cardiomyocytes (N = 3, n = 6 for IK_{sus} and N = 3, n = 5 for IK_{ss}). Data are represented as means \pm SE. C: comparison of the 8-CPTAM effect on IK_{sus} (left) and IK_{ss} (right) in non-pretreated cells and cells pretreated with each CaMKII inhibitor. For statistical analysis, paired t test were used for IK_{sus} analysis and Wilcoxon were used for IK_{ss}. Unpaired t tests were used to compare effects in (C). *P < 0.05, **P < 0.01, and ***P < 0.001.

Experimental Approach

We used pharmacological tools in freshly isolated rat ventricular myocytes to identify putative actors involved in EPAC dependent AP lengthening and K_p current decrease. An alternative approach with gene silencing would have required 2–3 days of cell culture likely to alter ion channels and signaling pathway (41). Moreover, EPAC knockout models exhibit some discrepancies about EPAC effect in cardiac function (17, 20) attributable to the different genetic background of the animals. Considering these discrepancies, a pharmacological approach in the non-consanguineous Wistar rat model seems to be physiologically relevant to study EPAC-induced regulation.

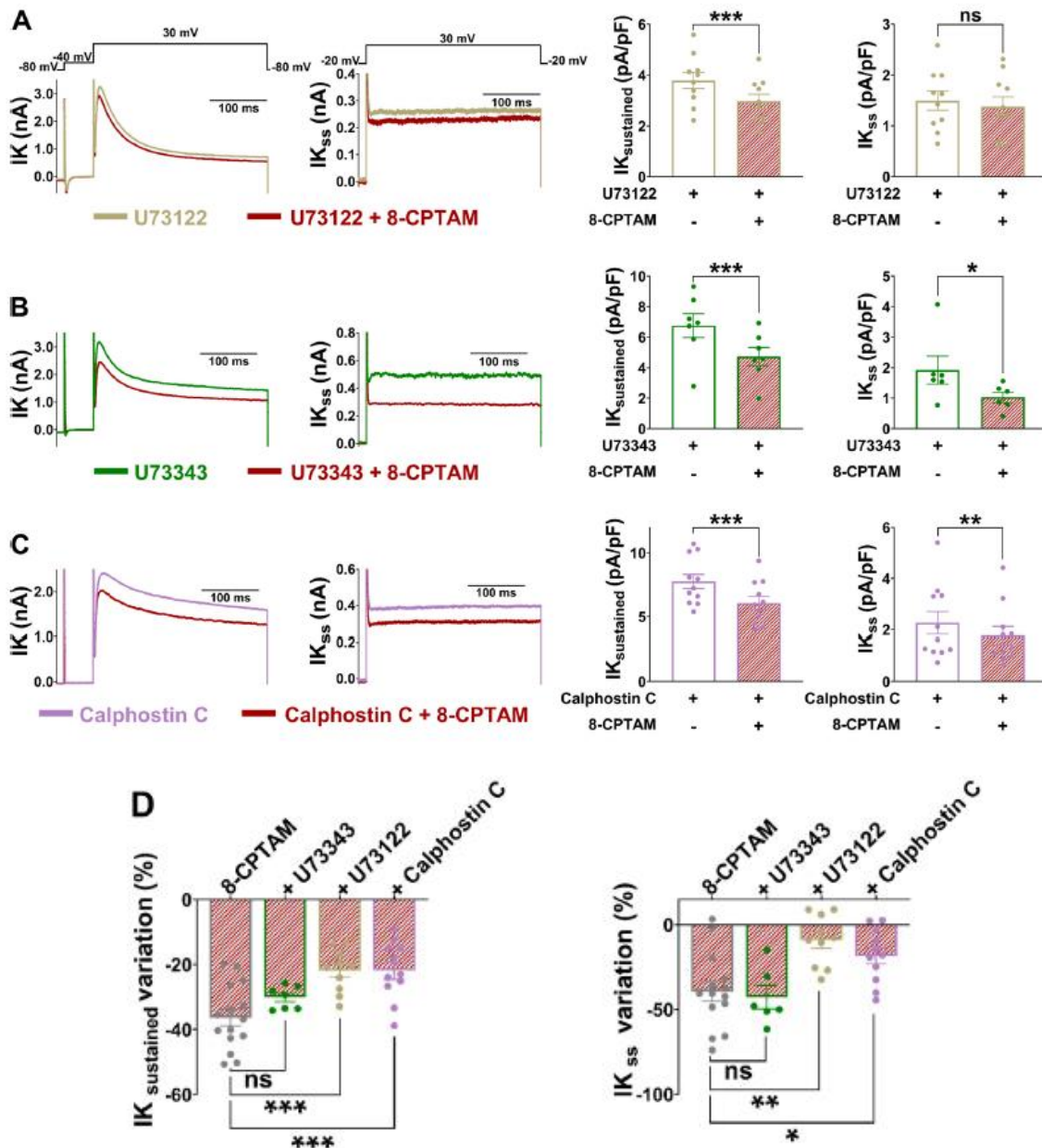
However, it is well known that pharmacological tools have inherent selectivity and specificity limitations. To minimize the limitations of pharmacological agents and ensure good delivery into cells, the inhibitors were systematically preincubated during at least 1 h and provided by perfusion at least 15min after reaching the ruptured configuration. In addition, they were included in the pipette when possible (see Supplemental Table S1).

Using pharmacological tools to elucidate the role of EPAC1 and EPAC2 in K^+ currents modulation, we cannot exclude selectivity and specificity issues. We conducted a thorough and comprehensive review of the primary literature, as recommended in a recent review (42).

Blockers of EPAC1 and EPAC2 have been well characterized. R-Ce3F4 is a noncompetitive inhibitor binding the nucleotide-binding domain of EPAC1 with 10-fold selectivity for EPAC1 (IC_{50} \approx 4.2 μ mol/L) over EPAC2 (IC_{50} \approx 44 μ mol/L) (43, 44). ESI-05 is a selective EPAC2 blocker by inhibiting cAMP-mediated EPAC2 GEF activity with apparent IC_{50} of 0.43 μ mol/L without suppressing EPAC1 GEF activity (45).

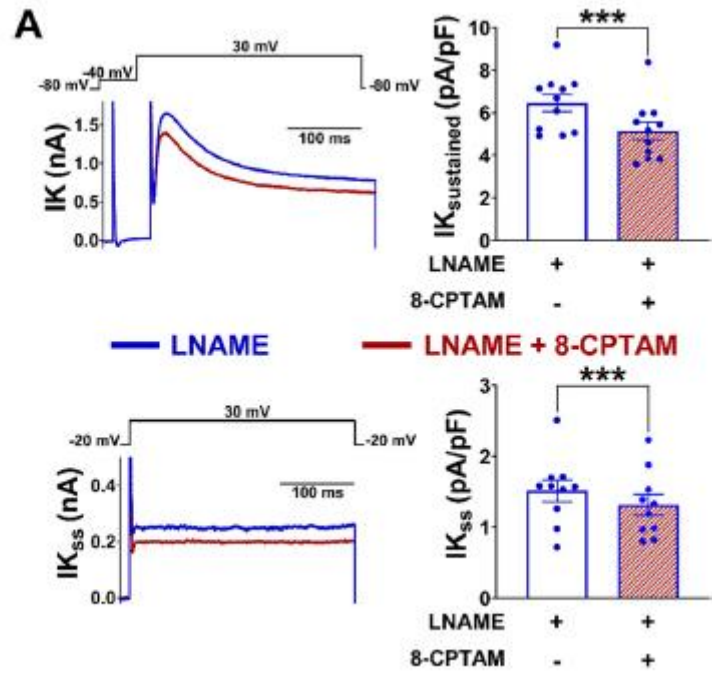
We encountered some problems when we used ESI-09, an inhibitor of both EPAC1 and EPAC2 (46). When cells were pre-exposed to ESI-09, recordings of action potentials were not possible with the cells becoming very unstable (data not shown). This suggests nonspecific effects, including proteins denaturation as previously described (47). With deep investigation, we found that similar results have been described in neurons (DRG) in a PhD dissertation (48). ESI-09 induced a silencing of dorsal root ganglion (DRG) neurons. Therefore, ESI-09 cannot be used in current clamp experiments in excitable cells. It appears to be relevant in some voltage-clamp experiments (49, 50), but we have preferred not using it and warn the physiological community about the use of ESI-09.

Figure 5. Phospholipase C/protein kinase C signaling pathway is involved in exchange proteins directly activated by cAMP (EPAC) signaling. A–C: effects of EPAC pharmacological activation on sustained component of potassium current (IK_{sus}) and steady-state potassium current (IK_{SS}) in cells pretreated with 5 μ mol/L of the selective PLC inhibitor U73122 ($N = 3$, $n = 10$), 5 μ mol/L of its inactive analogue U73343 ($N = 3$, $n = 7$) and the 2 μ mol/L of the PKC inhibitor Calphostin C ($N = 3$, $n = 11$). Mean \pm SE from current amplitudes are presented in the bar graphs. D: relative effect of the different inhibitors in 8-(4-chlorophenylthio)-20-O-methyl-cAMP acetoxymethyl ester (8-CPTAM)-dependent IK_{sus} and IK_{SS} modulation are shown by the means \pm SE of the percentage of variation. Data were analyzed with paired t tests in (A) and for the IK_{sus} component in (C), and with Wilcoxon test in (B) and for the IK_{SS} component in (C). In (D), unpaired t tests were used. * $P < 0.05$; ** $P < 0.01$, and *** $P < 0.001$.



Role of EPAC in Cardiac Physiology

Here we describe a new role for EPAC pathway in the modulation of cardiac physiology. Such modulation is relevant *in vivo*, e.g., fight or flight mechanisms with catecholamines increase (13). The aim is to increase the heart's contractile capacity by adapting its rate, cardiomyocyte calcium homeostasis, and metabolism, thereby increasing cardiac workload to adapt blood pressure and meet organ needs. In this sense, EPAC is likely to participate in the positive inotropic effect induced by catecholamines, since their activation modulates the excitation-contraction coupling of cardiomyocytes. In addition to the modulation of K^+ currents described in this study, it has been shown previously that following the application of acute or chronic 8-CPT, the amplitude of calcium transients is increased in cardiomyocytes (18, 19, 51). In parallel, EPAC activation increases cardiomyocyte cell shortening by sensitizing myofilaments to Ca^{2+} through phosphorylation of TnI and cMyBP-C (23).



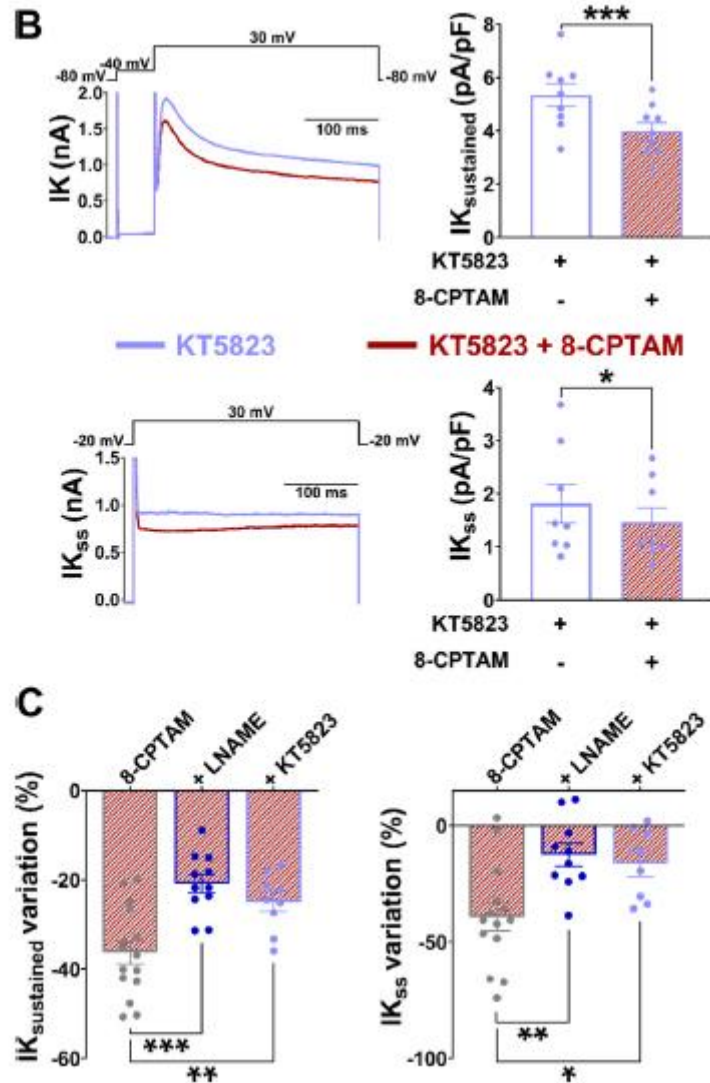


Figure 6. Involvement of nitric oxide (NO)/PKG pathway in exchange proteins directly activated by cAMP (EPAC)-mediated downregulation of K^+ currents. A: involvement of nitric oxide synthase (NOS) with representative traces and means \pm SE of sustained component of potassium current ($I_{K_{sust}}$) ($N = 4$, $n = 11$) and steady-state potassium current ($I_{K_{ss}}$) ($N = 4$, $n = 10$) subsequently to 8-(4-chlorophenylthio)-20-O-methyl-cAMP acetoxymethyl ester (8-CPTAM) treatment in the presence of 1 mmol/L of the NOS inhibitor nitro-L-arginine methyl ester (L-NAME) in extracellular and intrapipette media. B: implication of PKG depicted with representative traces and means \pm SE of $I_{K_{sust}}$ ($N = 3$, $n = 9$) and $I_{K_{ss}}$ ($N = 3$, $n = 9$) consecutively to 8-CPTAM stimulation in the presence of 1 μ mol/L of the PKG inhibitor KT5823. C: comparison of the relative effect of NOS and PKG inhibitions in 8-CPTAM-induced modulation of $I_{K_{sust}}$ and $I_{K_{ss}}$. Paired t tests were used for statistical comparisons of $I_{K_{sust}}$ and $I_{K_{ss}}$ for the KT5823 condition. Wilcoxon test was used for $I_{K_{ss}}$ comparison in L-NAME condition. Unpaired t tests were used to compare effects in C. * $P < 0.05$; ** $P < 0.01$, and *** $P < 0.001$.

EPAC Activation and Electrophysiological Properties on Cardiomyocytes

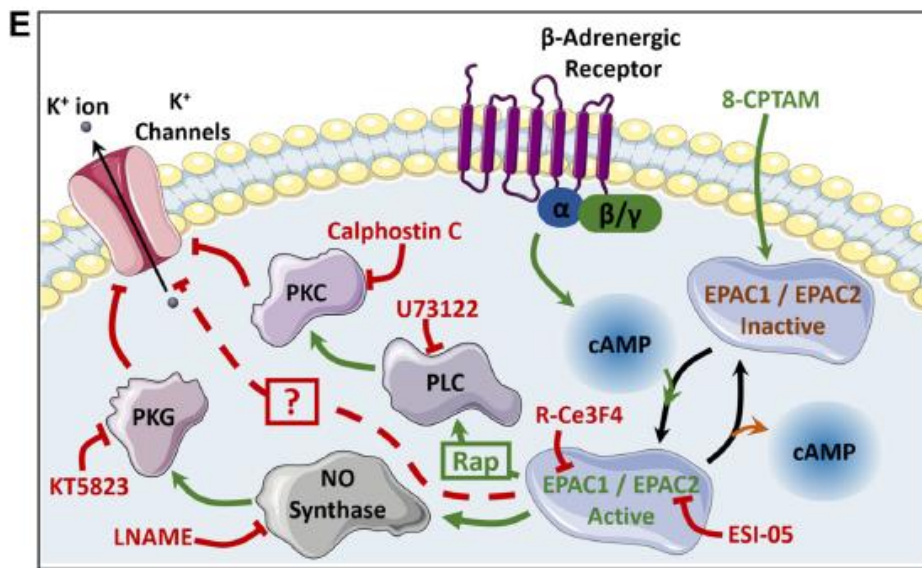
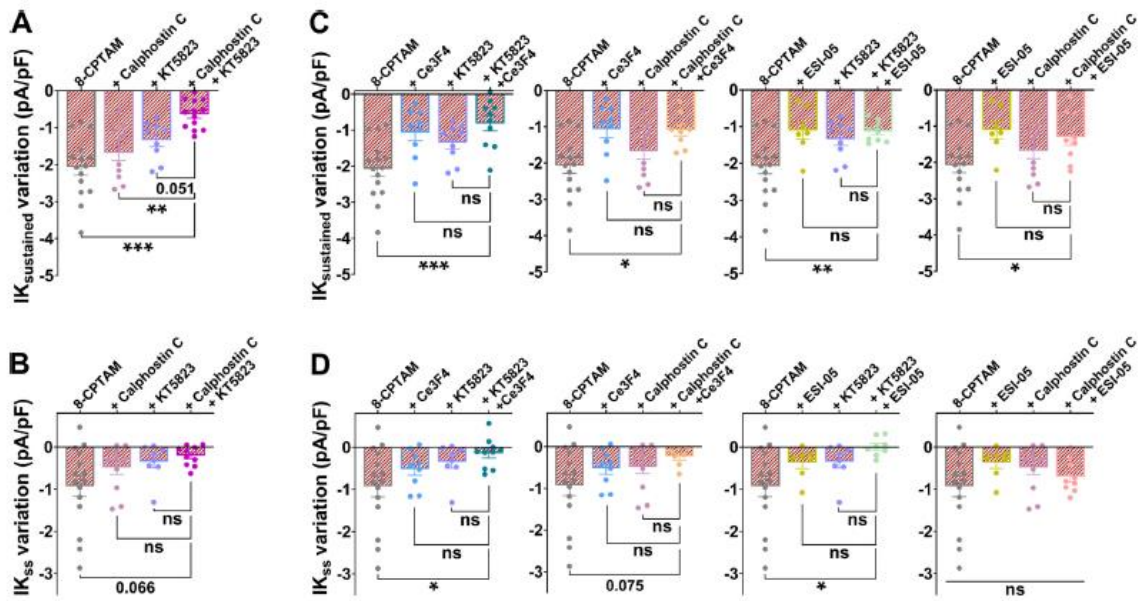
Previous work on EPAC in heart showed that it activates prohypertrophic pathways leading to cardiomyocyte hypertrophy (52, 53). Other deleterious effects of EPAC are ventricular arrhythmias, first described in vitro, and confirmed in transgenic mice with EPAC1 or EPAC2 gene disruption (54, 55). These responses could be explained by the fact that

EPAC produces AP lengthening in rat ventricular cardiomyocytes, which was confirmed by our present results (13). As a step forward, we show here that both EPAC1 and EPAC2 are involved in this AP prolongation. Importantly, β -adrenergic stimulation, in the presence of a PKA inhibitor, was reported to mimic the effects of EPAC activation with 8-CPTAM on APD (13) suggesting that pharmacological activation of EPAC is physiologically relevant. A similar effect on APD was also recently reported in both cellular and tissue atrial preparations from mice (11). However, using the intracellular microelectrode technique, Valli et al. (12) did not observe any effect of a non-AM 8-CPT form (used at 1 $\mu\text{mol/L}$) on APD₉₀ in mouse atrial or ventricle myocytes. This discrepancy may be due to the fact that we used here 10 $\mu\text{mol/L}$ of an AM form of 8-CPT. It has been demonstrated that the potency of 8-CPTAM was 100- to 1,000-fold greater than that of 8-CPT where the action of 1 $\mu\text{mol/L}$ 8-CPTAM exceeded that of 100 $\mu\text{mol/L}$ non-AM 8-CPT (56). Interestingly, AP prolongation has also been reported following a sustained activation of EPAC in isolated guinea pig left ventricular myocyte (16). Altogether, EPAC might be involved in ventricular arrhythmias since an increase in APD is a process that is correlated with the genesis of arrhythmia by predisposing cardiomyocytes to EADs and dispersion of repolarization (14).

The AP waveform is a fine balance between inward (depolarizing) and outward (repolarizing) currents among which Na⁺, Ca²⁺, and K⁺ currents are the main contributors. A decrease in I_{Na} peak magnitude was described in heart upon EPAC activation but this effect would be more related to reduced conduction rather than APD alteration (8, 12). I_{Ca} peak amplitude does not appear to be affected by EPAC (9, 13) even if an increase of I_{Ca,L} window current was reported when EPAC was activated by sustained application of 8-CPT (51). Thus, the main candidate involved in APD prolongation seems to be K_p current. The rapidly activating and inactivating component of K_p current carrying by I_{to} in rat ventricle is not modulated whereas the non-inactivating component (I_{Ksus}) is reduced by EPAC activation (13). This latter effect is likely to explain, at least in part, the reported APD lengthening. In rat ventricular cardiomyocytes, I_{Ksus} is itself a combination of several K_v currents distinguished kinetically and pharmacologically including a delayed-rectifier-like current (I_K) and a steady-state current (I_{KSS}) (57, 58). Brette et al. (13) reported a decrease of I_{KSS} and of I_{Ksus} upon EPAC activation while I_K appeared non-affected. That is why we focused our study on both currents. Using the specific inhibitors of EPAC1 or EPAC2, we show that both EPAC isoforms participate in I_{KSS} and I_{Ksus} inhibition following 8-CPTAM application which explains the APD prolongation. However, K_p current inhibition may not be the only mechanism of APD increase since EPAC activation potentializes the persistent component of I_{Na} (I_{Na, Late}) (15), a well-known contributor in reshaping the cardiac AP with arrhythmogenic potential under elevated sympathetic stimulation.

Figure 7. PKC- and PKG-dependent signaling pathways are independently involved in exchange proteins directly activated by cAMP (EPAC) signaling, but not preferentially stimulated by EPAC isoforms. The effect of different combinations of inhibitors can be shown in the different panels. A and B: effect of the concomitant inhibition of both PKC and PKG with Calphostin C (2 $\mu\text{mol/L}$) and KT5823 (1 $\mu\text{mol/L}$) on 8-(4-chlorophenylthio)-20-O-methyl-cAMP acetoxymethyl ester (8-CPTAM)-induced modulation of sustained component of potassium current (I_{Ksus}) (N = 5, n = 13) and steady-state potassium current (I_{Kss}) (N = 5, n = 10), respectively. C and D: impact of the different combinations of EPAC inhibitors (R-Ce3F4 at 25 $\mu\text{mol/L}$ or ESI-05 at 25 $\mu\text{mol/L}$) with PKC and PKG inhibitors (Calphostin C at 2 $\mu\text{mol/L}$ or KT5823 at 1 $\mu\text{mol/L}$) in EPAC-dependent modulation of I_{Ksus} and I_{Kss}, respectively. The effectives are: N = 3, n = 9 for each currents for the combination of R-Ce3F4 and KT5823; N = 3, n = 9 for I_{Ksus} and N = 3, n = 7 for I_{Kss} for the R-Ce3F4 and Calphostin C combination; N = 4, N = 9 for each currents for the ESI-05 and KT5823 combination; N = 4, n = 9 for each current for the ESI-05 and Calphostin C combination. One-way ANOVA with Holm-Sidak post hoc were used to compare the effects of the different combinations with the effect of single treatments in I_{Ksus} in all the combinations. Effects of the different combinations on I_{Kss} were compared to single treatments effects with

Kruskal–Wallis tests with Dunn’s post hoc, except for the R-Ce3F4 and KT5823 combination, for which one way ANOVA with Holm–Sidak post hoc was used. *P < 0.05, **P < 0.01, and ***P < 0.001.



EPAC Signaling Pathways Involved in K1 Current Modulation

To the best of our knowledge, nothing is known regarding the regulation of I_{KSS} by physiological signaling pathways. Among hypotheses, the ability of EPAC to activate CaMKII has been demonstrated (6, 7, 9, 15, 18, 19, 22). This CaMKII activation was attributed to a PLC-dependent pathway (6, 18) and its downstream effector, PKC (17, 18). Alternatively, CaMKII might be activated by S-nitrosylation due to NOS activity following selective EPAC activation by 8-CPT (21). In this study, we used two compounds (AIP and KN-93) with different structures and mechanisms of action to block CaMKII. Surprisingly, neither of them altered the effect of EPAC activation on I_{Kp} current suggesting that CaMKII is not involved. This result is in line with the fact that I_{to} , which is a well-known target for CaMKII (53), is not affected by EPAC activation with 8-CPTAM. Inhibition of CaMKII accelerates I_{to} inactivation in human atrial and rat ventricular myocytes (59, 60). The fact that

no effect was measured upon EPAC activation on Ito [(13) and Fig. 2, A and C] similarly suggests that CaMKII is not involved in the observed effect. Moreover, it is well documented that EPAC activation leads to Ca²⁺ leak from sarcoplasmic reticulum in a CaMKII-dependent mechanism via RyR phosphorylation (6, 17, 19, 21, 22) and/ or via IP₃-dependent opening of IP₃R (6). Thus, we examined the Ca²⁺ sensitivity of the EPAC-mediated inhibition of IK_s and IK_{SS} by inclusion of the fast Ca²⁺-specific chelator BAPTA (10 μmol/L) in the pipette-filling solution. Indeed, BAPTA is fast enough to chelate local Ca²⁺ released from the sarcoplasmic reticulum occurring in a restricted space whereas EGTA is not (61). The effect of 8-CPTAM on K⁺ channels activity was not altered (Supplemental Fig. S3) suggesting that EPAC-mediated channel inhibition does not rely upon an increase in subsarcolemmal Ca²⁺. Consequently, these data show a Ca²⁺- and CaMKII- independent effect and suggest that EPAC dependent Ca²⁺ handling and EPAC-mediated IK_{SS} modulation occur in different restricted microdomains.

Our results point to the involvement of two independent pathways, PLC/PKC and NOS/PKG, in the modulation of IK_s by 8-CPTAM (Figs. 5 and 6). The PLC/PKC pathway during EPAC activation is well documented (6, 9, 17–19, 21, 22). Alternatively, NOS has already been described as a downstream EPAC effector but its effect was attributed to CaMKII activation by S-nitrosylation (21). Here, we highlight the involvement of PKG in EPAC signaling which might be activated by cGMP production following NOS stimulation. Whether PKC and PKG exert their effect on ion channels carrying IK_s directly or indirectly remains to be determined. This is challenged by the fact that IK_s is a combination of several K_v currents for which molecular determinants remain incompletely understood. Moreover, two-pore-domain potassium channels (K₂P channel) such as TASK-1 and TREK-1 could contribute to the steady-state outward current in rat cardiac ventricular muscle (58, 62). To clarify these issues, further studies focusing on individual regulation of each current/ channel by EPAC are needed.

Because two pathways appear to be stimulated by EPAC leading to activation of PKC and PKG, it was tempting to speculate on putative preferential pathways induced by EPAC1 and EPAC2, respectively. In our experimental conditions, we failed to show a cumulative effect of the combination of one kinase inhibitor associated with one EPAC inhibitor suggesting that EPAC1 and EPAC2 do not discriminate a preferential pathway and they are able to activate both PLC/PKG and NO/ PKG pathways. In addition, blocking both pathways does not completely block the effect of 8-CPTAM suggesting that a third pathway might be involved. Further studies are needed to characterize the remaining missing link (Fig. 7E).

Our results should be considered alongside the recent study published by Sartre et al. (63). They showed that EPAC1 may have at least four activation states, depending on its interaction with lipid membranes, cAMP presence or both (63). Specifically, soluble EPAC1 is seemingly not activated at the low cAMP concentrations encountered in the cytosol while liposomal membrane-linked EPAC1 is highly sensitive to cAMP rate. According to the fact that EPAC1 is preferentially localized around the nucleus and can be exported to the plasma membrane by cAMP binding (64, 65), whether 8-CPTAM is able to mimic the cAMP-dependent trafficking of EPAC1 to the sarcolemma in living cells remains to be investigated. However, acute 8-CPTAM treatment has been described to perfectly mimic the effect of a β-adrenergic-induced EPAC activation in APD modulation in rat ventricular myocytes (13).

Perspectives and Significance

Taken together, these data provide a solid foundation for research to further explore the physiological role of EPAC in cardiac myocytes, but also other excitable and nonexcitable cells. In this era of translational research (66), most of the focus has been on pathophysiological condition. EPAC has been implicated in myocyte Ca^{2+} homeostasis perturbations leading to cardiomyocyte hypertrophy (4–6) and proarrhythmic effects (7, 8). EPAC-dependent proarrhythmic activity was reported as a decrease in conduction velocity in both whole heart (8) and atria (11). At the cellular level, EPAC activation can promote arrhythmias by increasing the likelihood of DAD trigger following $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger activation by diastolic Ca^{2+} leak from sarcoplasmic reticulum (8). In addition, EPAC activation may be the cause of EAD by reshaping and lengthening action potential via an increase of the persistent component of the fast Na^{+} current (15) and/or a decrease of K^{+} current (13, 16). DAD and EAD are recognized as major source of ectopic activity (10, 14). Thus, a growing number of publications suggest that EPAC could be a new therapeutic target to prevent arrhythmias under pathological conditions such as hypertrophy.

DATA AVAILABILITY

The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.B., F.B., and L.S. conceived and designed research; A.B., O.R., T.H., A.L., L.B., S.d., C.S., F.B., and L.S. performed experiments; A.B., O.R., A.L., and L.S. analyzed data; A.B., O.R., A.L., and L.S. interpreted results of experiments; A.B. prepared figures; A.B., F.B. and L.S. drafted manuscript; A.B., O.R., A.F., C.S., R.G., F.B., and L.S. edited and revised manuscript; A.B., O.R., A.L., L.B., S.d., A.F., C.S., R.G., F.B., and L.S. approved final version of manuscript.

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