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Protective role of soluble adenylyl cyclase against reperfusion-induced injury of cardiac cells

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ABSTRACT

Aims: Disturbance of mitochondrial function significantly contributes to the myocardial injury that occurs during reperfusion. Increasing evidence suggests a role of intra-mitochondrial cyclic AMP (cAMP) signaling in promoting respiration and ATP synthesis. Mitochondrial levels of cAMP are controlled by type 10 soluble adenylyl cyclase (sAC) and phosphodiesterase 2 (PDE2), however their role in the reperfusion-induced injury remains unknown. Here we aimed to examine whether sAC may support cardiomyocyte survival during reperfusion.

Methods and results: Adult rat cardiomyocytes or rat cardiac H9C2 cells were subjected to metabolic inhibition and recovery as a model of simulated ischemia and reperfusion. Cytosolic Ca^{2+}, pH, mitochondrial cAMP (live-cell imaging), and cell viability were analyzed during a 15-min period of reperfusion. Suppression of sAC activity in cardiomyocytes and H9C2 cells, either by sAC knockdown, by pharmacological inhibition or by withdrawal of bicarbonate, a natural sAC activator, compromised cell viability and recovery of cytosolic Ca^{2+} homeostasis during reperfusion. Contrariwise, overexpression of mitochondria-targeted sAC in H9C2 cells suppressed reperfusion-induced cell death. Analyzing cAMP concentration in mitochondrial matrix we found that inhibition of PDE2, a predominant mitochondria-localized PDE isoform in mammals, during reperfusion significantly increased cAMP level in mitochondrial matrix, but not in cytosol. Accordingly, PDE2 inhibition attenuated reperfusion-induced cardiomyocyte death and improved recovery of the cytosolic Ca^{2+} homeostasis.

Conclusion: sAC plays an essential role in supporting cardiomyocytes viability during reperfusion. Elevation of mitochondrial cAMP pool either by sAC overexpression or by PDE2 inhibition beneficially affects cardiomyocyte survival during reperfusion.

1. Introduction

Myocardial reperfusion, after a prolonged ischemic period, is associated with biochemical and ultrastructural changes that can lead to irreversible tissue injury [1]. Prevention of the reperfusion-induced injury is still a challenging therapeutic goal for cardiologists. Therefore, an understanding of the mechanisms involved in reperfusion-induced injury may lead to the development of better techniques and a better outcome of ischemia/reperfusion therapy.

A large body of literature suggests a role of mitochondrial dysfunction in reperfusion-induced injury. There are two main patterns of cardiomyocyte death during reperfusion due to mitochondrial dysfunction: (1) acute necrotic cell death and (2) delayed apoptotic cell death. The necrotic cell death occurs during a narrow window of reperfusion. Several mechanisms have been proposed to be responsible for the mitochondrial injury during this period, including excessive Ca^{2+} accumulation, mitochondrial permeability transition pore opening, and reactive oxygen species formation [2]. Accordingly, diverse strategies have been applied to protect/support mitochondrial function during the initial phase of reperfusion and have demonstrated significant amelioration of reperfusion-induced injury [3].

Among several signaling pathways controlling mitochondrial

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function, intra-mitochondrial cAMP signaling has recently received great attention. Several reports emphasized an evolutionarily conserved role for mitochondrial cAMP/cAMP-dependent protein kinase (PKA) signaling in controlling mitochondrial respiration and ATP synthesis in mammals [4,5] and yeast [6] (for review see [7]). Although the plasmalemmal adenylyl cyclase was initially assumed to be the source of mitochondrial cAMP, a recent study [8] reconsidered this paradigm demonstrating that the mitochondrial membrane is impermeable to cytosolic cAMP and a mitochondria-localized cAMP source is required. The type 10 soluble adenylyl cyclase (sAC) is localized in mitochondria and synthesizes cAMP in the mitochondrial matrix [8,9]. cAMP produced in mitochondria promotes cytochrome *c* oxidase activity via PKA-dependent phosphorylation of subunit IV [5]. As a result, activation of the mitochondrial sAC/PKA pathway promotes ATP synthesis and attenuates oxygen radical production [4,5]. It is worthy to mention that mitochondrial sAC is a bicarbonate and Ca^{2+} sensor and, therefore, couples the activity of the TCA cycle (the main source of CO_2/bicarbonate in the cell) as well as alterations in the intra-mitochondrial Ca^{2+} concentration to mitochondrial function [8]. Collectively, under basal conditions, the intra-mitochondrial cAMP signaling positively affects mitochondrial function; whether it may play such a role during reperfusion and, therefore, contribute to cardiomyocyte survival remains unknown and was the aim of the present study. Applying isolated adult rat cardiomyocytes and cardiac H9C2 cells subjected to metabolic inhibition and recovery, we provide evidences for the beneficial role of mitochondrial sAC in protection against reperfusion-induced cell death. In addition, supporting mitochondrial cAMP signaling by specific inhibition of PDE2 significantly attenuates necrotic cell death during reperfusion.

2. Methods

2.1. Cardiomyocyte isolation and culture

The investigation conforms to the German animal welfare laws and was approved by the Animal Welfare Officer of the Justus Liebig University Giessen. The animal housing facility was licensed by the local authorities (Nr. FD62-§11JLUHumPhys). The methods used to euthanize the rats were consistent with the recommendations of the AVMA Guidelines for the Euthanasia of Animals. Briefly, 200–300 g male Wistar rats were euthanized by 5% isoflurane followed by cervical dislocation. Ventricular cardiomyocytes were isolated by perfusion of hearts with collagenase type II (300 U/ml) and cultured as previously described [10]. Cells were used for experiments 6 h after isolation.

2.2. H9C2 cell culture

Rat embryonic cardiomyoblast cells (cell line H9C2; ATCC, CRL-1446) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1 mmol/l sodium pyruvate, and 1% penicillin/streptomycin. One day before experiments FCS was reduced to 3%.

2.3. Experimental protocols

All normoxic experiments were performed in standard medium (in mmol/l: 96 NaCl, 2.6 KCl, 1.2 $KH2PO_4$, 1.2 $MgSO_4$, 22 $NaHCO_3$, 25 HEPES, 1.2 $CaCl_2$, 5 glucose; pH 7.4) equilibrated with 95% air and 5% CO_2 at 36 °C. For normoxic experiments with H9C2 cells, the standard medium was supplemented with 2 mmol/l pyruvate.

To simulate ischemia in cardiomyocytes, metabolic inhibition (chemical anoxia, CA) was applied using 2 mmol/l sodium cyanide (NaCN) in glucose-free standard medium at pH 6.4. CA in H9C2 cells was performed in glucose- and pyruvate-free standard medium (pH 7.4) supplemented with 2 mmol/l NaCN and 5 mmol/l 2-deoxy-D-glucose (2-DOG) at 36 °C. Reperfusion (R) in both cell types was performed with standard medium.

2.4. Determination of cytosolic Ca^{2+}

The temperature-controlled perfusion chamber (36 °C) was mounted on a microscope (Olympus IX-70, Germany) adapted to a video-imaging system (Visitron Systems, Germany) containing a monochromator for excitation and a CCD camera (Retiga 2000-RV, Canada) for fluorescence detection. Fluorescence data were analyzed using VisiView software (Visitron Systems, Germany). The fluorescent signal was recorded continuously every 3 s throughout the experiment except for measurements made during reperfusion of cardiomyocytes, which were performed every 0.3 s.

To measure cytosolic Ca^{2+} concentrations, cardiomyocytes and H9C2 cells were loaded at 37 °C with the acetoxymethyl ester of fura-2 (2.5 μmol/l) for 45 min and then washed twice and further incubated for 20 min in culture medium at 37 °C. The loading protocol used was selected from a number of variations because it provides the highest yield in fluorescence with the least amount of dye compartmentation (compartmentation: 9.1 ± 0.9% in cardiomyocytes and 13.8 ± 1.9% in H9C2 cells). The dye compartmentation was defined as described previously [11]. The fluorescence from fura-2-loaded cells was at least 10 times higher than background fluorescence. Fura-2 loaded cells were excited at 340 and 380 nm. Cytosolic Ca^{2+} concentration was expressed as the fura-2 emission (510 nm) ratio after excitation at 340 nm or 380 nm.

2.5. Detection of cytosolic pH

Cardiomyocyte cytosolic pH was measured with the fluorescent dye BCECF as previously described [11]. Briefly, cells were loaded with the acetoxymethyl ester of BCECF (0.5 μmol/l) for 5 min at 35 °C, then washed twice and further incubated for 30 min in culture medium at 37 °C. The dye was excited at 440 nm and 490 nm and emission was measured at 535 nm. The pH was expressed as the BCECF emission ratio after excitation at 490 nm and 440 nm. BCECF ratio calibration was performed in medium containing the following (in mmol/l): 10 NaCl, 125 KCl, 1.2 $MgSO_4$, 25 HEPES, 0.5 EGTA, 5 glucose, 1 μmol/l nigericin and 10 μmol/l valinomycin, at different pH (6.0, 6.5, 7.0, 7.5) and at 36 °C. BCECF fluorescence was recorded continuously every 60 s during the experiment.

2.6. Determination of viability and necrosis

The viability of cardiomyocytes was determined as the percentage of cells able to maintain cytosolic Ca^{2+} homeostasis during reperfusion (Fig. 1). Necrosis in cardiomyocytes and H9C2 cells was determined 60 min after reperfusion by incubation of the cells with 1 μg/ml propidium iodide and 1 μg/ml 4′,6-diamidino-2-phenylindole for at least 1 min at 37 °C followed by fluorescent microscopy visualization. For quantitative assay, a blind analysis of 200 cells from four to five randomized fields was used.

2.7. Analysis of lactate dehydrogenase activity in culture medium

Lactate dehydrogenase (LDH) activity in the cell culture medium was used as an additional indicator of necrosis and was determined with a Cytotoxicity Detection Kit (Roche Applied Science). After each experiment the culture medium was collected and centrifuged at 800*g* for 5 min at 4 °C. The supernatant was used for LDH analysis using an ELISA reader.

2.8. Total cellular cAMP content

Analysis of the total cellular cAMP content was performed in cardiomyocytes using a cAMP (direct) Enzyme Immunoassay Kit (Enzo Life Sciences). The absorbance measured at 405 nm was used to calculate the concentration of cAMP by applying a calibration curve according to the manufacturer's instructions.

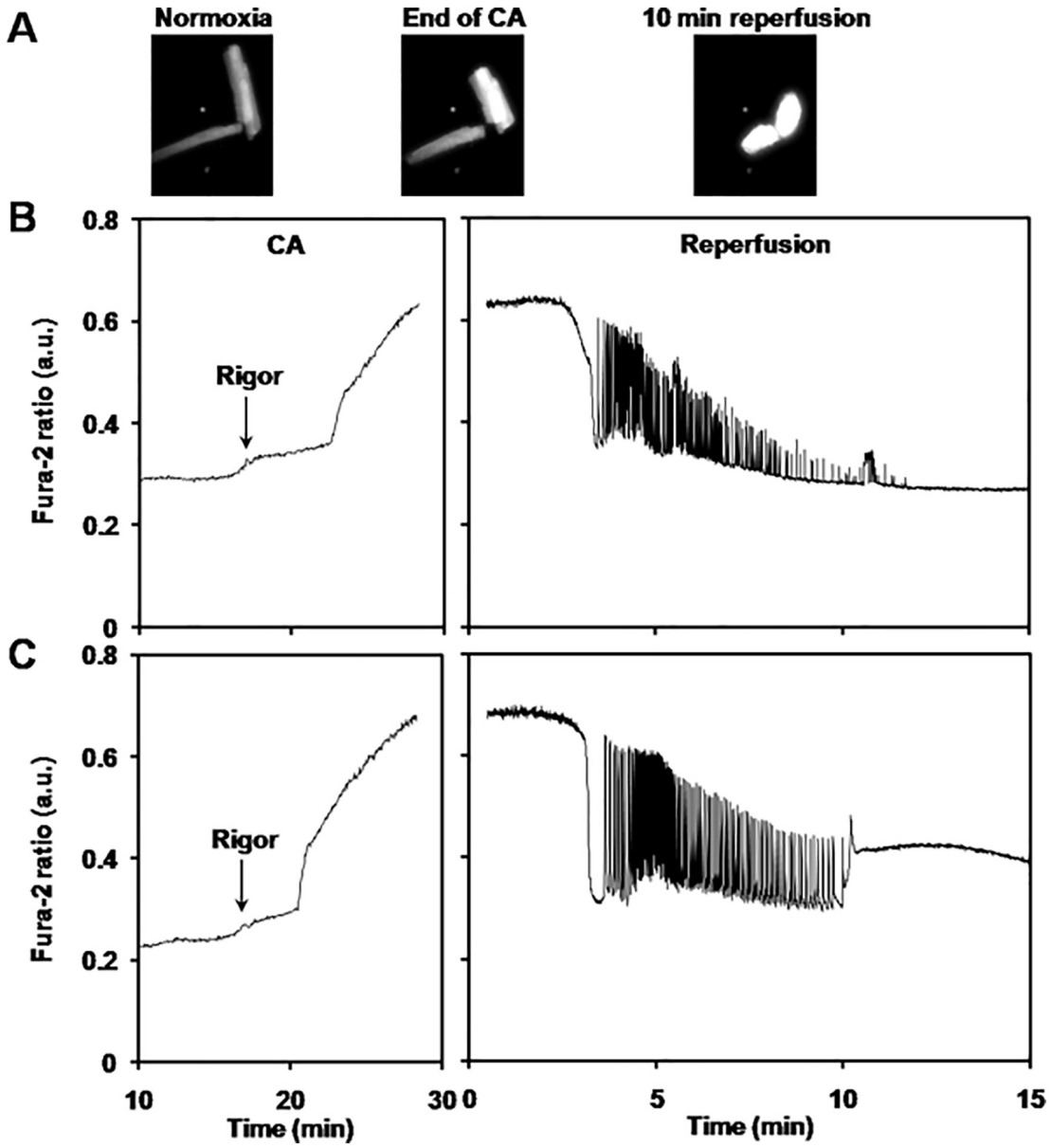

Fig. 1. Recovery of Ca^{2+} homeostasis in reperfused cardiomyocytes. (A) Representative Fura-2 fluorescent images (excitation at 340 nm) of adult rat cardiomyocytes before CA (Normoxia), at the end of CA, and after 10 min of reperfusion. Note the changes in cell length observed during CA (rigor contracture) and during reperfusion (hypercontracture). (B, C) Representative original recordings of cytosolic Ca^{2+} concentration (Fura-2 ratio, arbitrary units) during CA and reperfusion in a cell that recovered Ca^{2+} homeostasis during reperfusion (B) and in a cell that lost metabolic control leading to sustained Ca^{2+} overload after 10 min of reperfusion (C). Rigor indicates the time point of rigor contracture, i.e., complete energy depletion.

2.9. Fluorescence resonance energy transfer (FRET) analysis of cAMP

For FRET-based live imaging analysis, H9C2 cells were transfected with plasmids encoding corresponding sensor. Transfections were performed at 50–60% confluence by electroporation with the Amaxa™ Nucleofector™ II device (Lonza, Switzerland) using the T-20 program. To transfect 10^6 cells, 9 μg of total cDNA diluted in 100 μl of electroporation buffer (5 mmol/l KCl, 15 mmol/l $MgCl_2$, 50 mmol/l mannitol, 120 mmol/l Na_2HPO_4/NaH_2PO_4, pH = 7.2) was used. After electroporation, cells were seeded on 24-mm diameter glass coverslips and cultured in 10% FCS for 24 h followed by 24 h of serum starvation in DMEM supplemented with 3% FCS.

For determination of compartmentalized cAMP content, H9C2 cells were transfected with an EPAC-based cAMP sensor without (H30, for analysis of cytosolic cAMP) or with a mitochondrial targeted sequence (4mtH30, for analysis of mitochondrial cAMP), as previously described [8]. cAMP probes were excited at 430 nm and emission light was acquired at 470 nm for the CFP channel and at 530 nm for the YFP channel. Images were acquired with an inverted microscope (oil immersion objective 40×, Zeiss, Germany) and imaging system (Visitron, Germany) every 6 s. Analysis of the FRET signal was accomplished with VisiView software (Visitron, Germany) or ImageJ software. Emission signals obtained in the cell-free region (background) were subtracted from the corresponding emission signals obtained within the region of interest and presented as a CFP/YFP ratio.

Since the fluorescence ratio may be affected by the expression level of the probe, in all experiments the intensity of the basal CFP fluorescence was determined and the mean values between the groups were compared. No differences in mean values of the basal CFP intensity between the experimental groups were observed.

2.10. Soluble adenylyl cyclase overexpression

Plasmids for the transient expression of sAC, specifically, non-targeted, leading to the sAC expression predominantly in the cytosol (cyto-sAC), and mitochondria-targeted (mito-sAC), were kindly provided by Dr. R. Acin-Perez (Centro Nacional de Investigaciones Cardiovasculares, Madrid). H9C2 cell were transfected with plasmids using the jetPRIME transfection reagent (Polyplus) according to the manufacturer's instruction. Cells were seeded into 6-well plates, transfected with 2 μg DNA in cell culture medium M199 supplemented with 10% FCS, and cultured for 24 h followed by washing and 24 h serum starvation in medium with 3% FCS. Transfection with control, GFP-targeted plasmids shows 62% efficacy 24 h after transfection determined by FACS assay. Transfection with both sAC-encoded plasmids shows about two-fold increase of sAC expression (Supplementary Fig. 2A) and significant elevation of cellular cAMP (Supplementary Fig. 2C).

Mitochondrial localization of the mito-AC was confirmed by confocal microscopy (Supplementary Fig. 2B). For this purpose H9C2 cells growing in 8-chamber slides were transfected with 200 ng plasmids encoding HA-tagged mito-sAC per chamber using the jetPRIME transfection reagent (Polyplus). 2 days after transfection, mitochondria and nuclei were stained using MitoTracker Red CMXRos (200 nM) and Hoechst 33342 nuclear dye (2 μg/ml) for 20 min at 37 °C. Cells were then washed twice with PBS, fixed and permeabilized with 100% methanol for 20 min at −20 °C. Artificially expressed sAC was visualized by staining with anti-HA-tag primary antibody (1:300 dilution, Cell Signaling) and with FITC-labelled secondary antibody. Images were acquired with 63× oil-immersion objective using a Nikon spinning disk confocal microscope CSU-X and EMCCD camera iXon3 DU-888 Ultra.

2.11. Soluble adenylyl cyclase knockdown

For sAC knockdown experiments, cells were transfected either with scramble or with sAC shRNA encoding plasmids by electroporation using T-020 program in Amaxa Nucleofector II Device (Lonza). All downstream analyses were carried out 3 days after transfection. sAC shRNA (5′-GGGGTACCAAAAAAGTGGAAAGTGGAACGAAAGCATCTCTTGAATGCTTTCGTTCCACTTTCCACAAACAAGGCTTTTCTCCAAG-3′) was expressed by the U6 promoter and a randomized sequence (scramble) based on sAC shRNA sequence was used as a control. Hairpin loop in the sequence is underlined.

2.12. Western blotting

Cells were lysed and equal amounts of total proteins (20–30 μg/well) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Primary antibodies were used to stain the following proteins: sAC (clones R21, CEP Biotech, NY) and vinculin (Cell Signaling). After incubation with horseradish peroxidase-conjugated secondary antibodies, specific bands were visualized by chemiluminescence using an ECL Kit (Promega, Germany). Equal sample loading was confirmed by stripping the membranes with Restore Western blot stripping buffer followed by staining with anti-vinculin antibody.

2.13. Statistical analysis

Data are given as mean ± standard error of mean (SEM). Comparisons between two groups were performed using an unpaired Student's *t*-test. Comparisons of means between more than two groups were performed by one-way analysis of variance. Statistical significance was accepted when $P < 0.05$. Number (n) for all experiments refers to independent single measurements. For live imaging experiments, either with cardiomyocytes or with H9C2 cells, 4–8 cells were investigated for each single measurement.

3. Results

3.1. Inhibition of sAC potentiated reperfusion-induced injury in cardiomyocytes

Depletion of cellular ATP is a major damaging event during ischemic insult. Thus, to simulate ischemia, metabolic inhibition, defined here as a chemical anoxia (CA), was carried out by treatment of cultured adult rat cardiomyocytes with sodium cyanide in glucose-free medium. During CA cells became depleted of ATP within 25 min, as indicated by rigor contracture [12]. This event was followed by accumulation of Ca^{2+} in the cytosol (Fig. 1). Upon simulated reperfusion, the majority of cells rapidly recovered cytosolic Ca^{2+} homeostasis, which was usually accompanied by a short phase of spontaneous Ca^{2+} oscillations (Fig. 1B). Some cells, however, lost viability during this initial reperfusion phase, as indicated by abrupt cessation of Ca^{2+} oscillations and irreversible elevation of the basal Ca^{2+} concentration (Fig. 1C).

To examine the role of sAC in the recovery of Ca^{2+} homeostasis and cell viability, sAC activity was suppressed by treatment with the selective sAC inhibitor KH7 (Tocris, Germany) during reperfusion. Previous studies demonstrated that KH7 inhibits sAC in various cell types at a concentration range of 10–30 μmol/l, whereas it has no effect on the membrane-bound AC and soluble guanylyl cyclase up to 100 μmol/l [13–15]. Accordingly, in rat cardiomyocytes KH7 reduced total cellular cAMP content in a dose-dependent manner, with a significant effect (about 50%) observed at 30 μmol/l (Fig. 2A). Treatment of cardiomyocytes with 30 μmol/l KH7 during reperfusion markedly disturbed recovery of cytosolic Ca^{2+} homeostasis and led to the loss of cell viability (Fig. 2C–E). This was accompanied by elevated necrosis (Fig. 2F). In contrast, applying KH7 to cells after identical handling, but without metabolic inhibition (i.e., normoxic superfusion), had no effect on cytosolic Ca^{2+} levels or cell viability during reperfusion (Fig. 2B, D–F). Similarly to KH7, treatment with a novel sAC inhibitor LRE1 (Sigma-Aldrich) [16] reduced viability and induced cell death (Supplementary Fig. 1B, C). Thus, the activity of sAC in reperfused cardiomyocytes is crucial for cell survival.

To substantiate the role of sAC, we applied another approach to suppress the activity of sAC, i.e., depletion of bicarbonate concentration during reperfusion. Bicarbonate is a natural stimulator of sAC, and reduction of bicarbonate from the physiological level of 22 to 0 mmol/l has been found to reduce sAC activity by about 60% [17]. Accordingly, incubation of cardiomyocytes for 1 h in bicarbonate-free, HEPES-buffered medium significantly reduced total cellular cAMP content, which was comparable to direct sAC inhibition with KH7 (Fig. 3A). The absence of bicarbonate during CA/R significantly attenuated the recovery of Ca^{2+} homeostasis and reduced cell viability (Fig. 3).

It has been shown that cytosolic pH plays an important role in reperfusion-induced cardiomyocyte injury [18,19]. To examine whether alteration in cytosolic pH may be a cause for the detrimental effect of bicarbonate withdrawal, cytosolic pH was analyzed in cardiomyocytes during CA/R. There was no difference in cytosolic pH between the groups (Fig. 3C). Thus, together with pharmacological inhibition of sAC, these results argue for a beneficial role of sAC in reperfused cardiomyocytes.

3.2. Mitochondrial sAC supports cell viability during reperfusion

Previous reports emphasized the significance of intra-mitochondrial sAC in supporting mitochondrial function [6,8,20]. Therefore, we aimed to determine whether the activity of mitochondrial sAC plays a role in the recovery of mitochondrial function and in the protection of cardiomyocytes during reperfusion. For this purpose, the embryonic cardiomyocyte line H9C2 was used to achieve the mitochondrial overexpression of sAC.

As in adult rat cardiomyocytes, inhibition of sAC in H9C2 cells

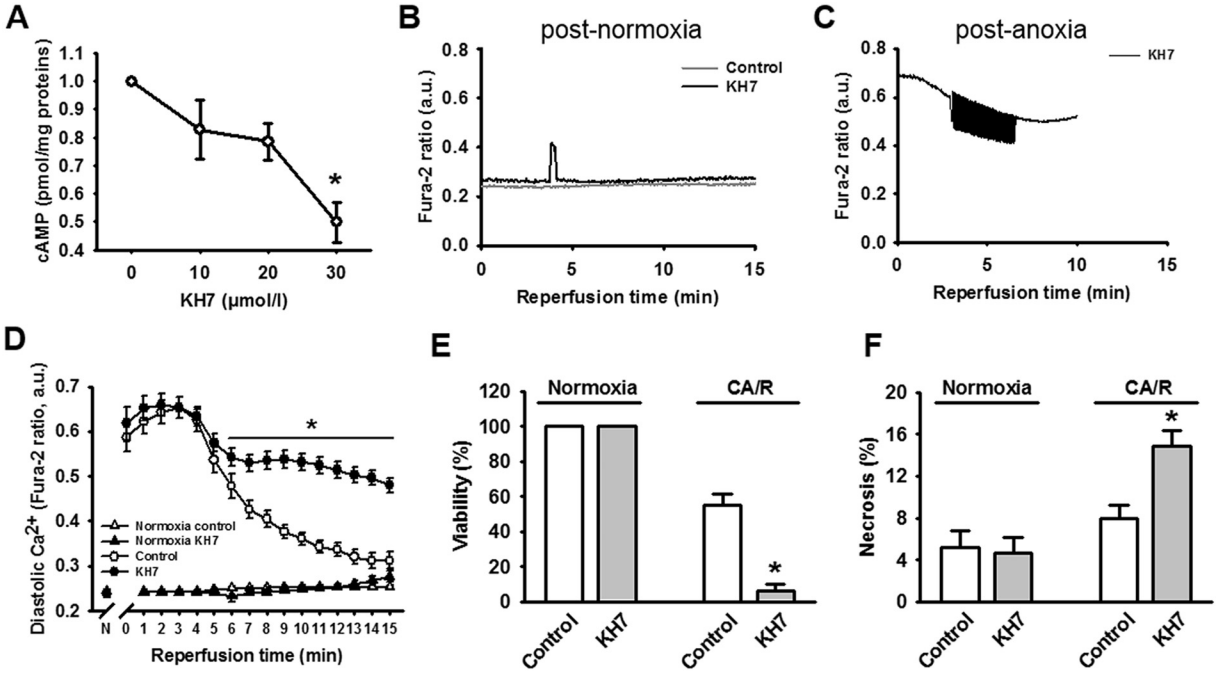

Fig. 2. Inhibition of sAC during reperfusion enhances reperfusion-induced injury in cardiomyocytes. (A) KH7 effect on the total cellular cAMP content after a 3-h treatment. Values are means ± SEM. $n = 8–14$. *$P < 0.05$ vs. 0, 10, or 20 μmol/l KH7. (B, C) Representative original recordings of cytosolic Ca^{2+} level (Fura-2 ratio, arbitrary units) in single cardiomyocytes during reperfusion without (Control) or with 30 μmol/l KH7 after 30 min incubation in normoxic (B) or CA (C) medium. (D) Time course of the diastolic, i.e., minimal, Ca^{2+} level (Fura-2 ratio, arbitrary units) in cardiomyocytes undergoing 30 min CA and 15 min reperfusion without (Control) or with 30 μmol/l KH7 only during reperfusion. The time course of diastolic Ca^{2+} level in cardiomyocytes reperfused after normoxic incubation (Normoxia) is also presented. "N" on the X-axis indicates normoxia just before CA. Values are means ± SEM. $n = 6–7$ for CA and $n = 3–5$ for normoxic incubation. *$P < 0.05$, KH7 vs. Control after CA. (E, F) Statistical analyses of cell viability during 10 min reperfusion and necrosis after 1 h of reperfusion. The treatment conditions are similar to those described for (D). Values are means ± SEM. $n = 5–7$ for (E) and $n = 4–13$ for (F). *$P < 0.05$ vs. Control after CA/R.

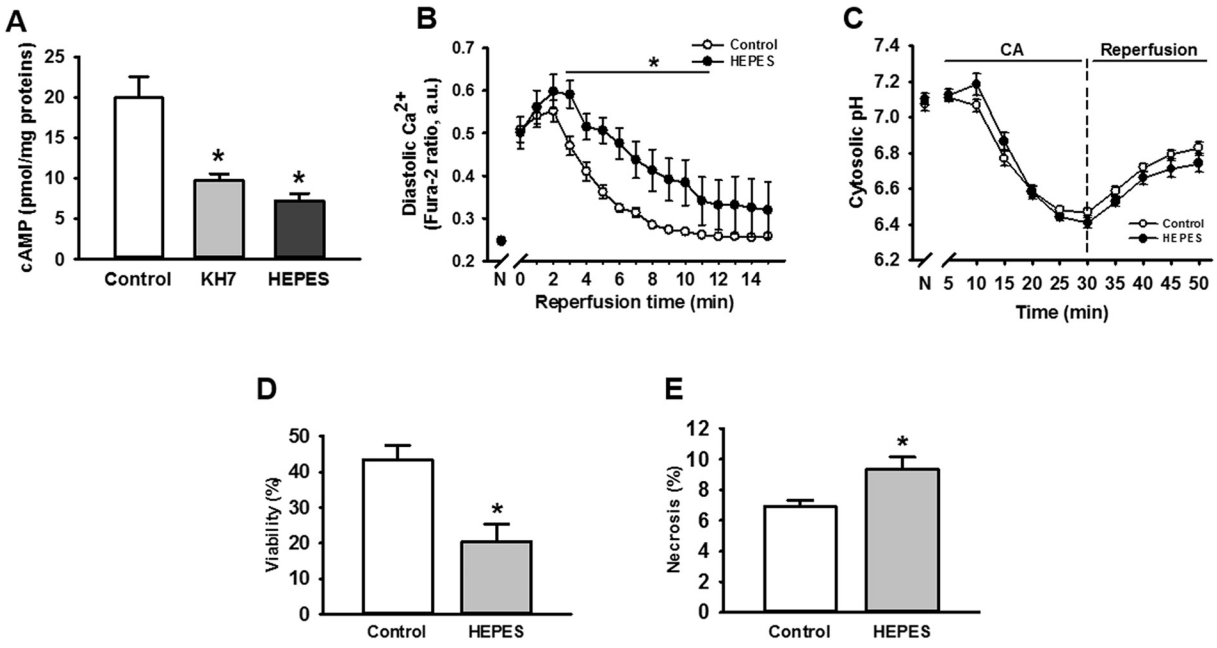

Fig. 3. Bicarbonate withdrawal exacerbates reperfusion-induced injury in cardiomyocytes. (A) Effect of the incubation of adult rat cardiomyocytes with 30 mmol/l KH7 or in bicarbonate-free medium (HEPES) for 1 h on the total cellular cAMP. Values are means ± SEM. $n = 3–4$. *$P < 0.05$ vs. Control. (B, C) Time course of the diastolic Ca^{2+} level (Fura-2 ratio, arbitrary units) and cytosolic pH in cardiomyocytes undergoing 30 min CA and 15 min reperfusion in bicarbonate-buffered (Control) or HEPES-buffered (HEPES) medium. "N" on the X-axis indicates normoxia just before CA. Values are means ± SEM. $n = 3–5$. *$P < 0.05$, KH7 vs. Control. (D, E) Statistical analyses of cell viability during 10 min reperfusion and necrosis after 1 h of reperfusion. The treatment conditions are similar to those described for (B,C). Values are means ± SEM. $n = 4$ for (D) and $n = 6–7$ for (E). *$P < 0.05$ vs. Control.

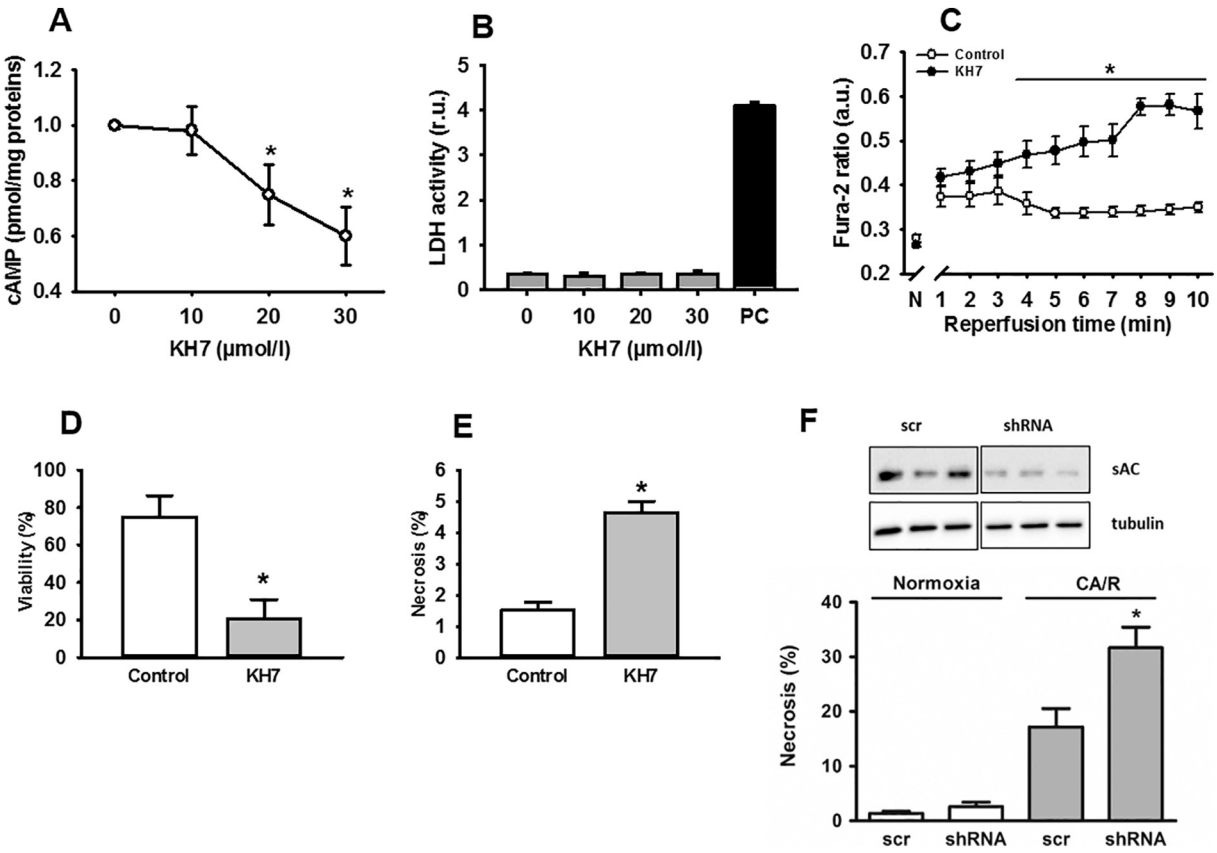

Fig. 4. Inhibition of sAC during reperfusion enhances reperfusion-induced injury of H9C2 cells. (A, B) Dose-dependent effect of KH7 on the total cellular cAMP content and lactate dehydrogenase (LDH) activity in the cell culture medium (presented as a ratio to individual protein content, relative units) after 3 h treatment. Data are means ± SEM. $n = 7$ for (A) and $n = 3$ for (B). "PC" in (B) indicates positive control, i.e., treatment with 0.05% Triton X-100 for 10 min at 4 °C to permeabilize the cells and release LDH. *$P < 0.05$, vs. 0 µmol/l KH7. (C) Time course of the diastolic Ca^{2+} level (Fura-2 ratio, arbitrary units) in H9C2 cells undergoing 30 min CA and 10 min reperfusion without (Control) or with 30 µmol/l KH7. Values are means ± SEM, $n = 7–8$. *$P < 0.05$, KH7 vs. Control. "N" on the X-axis indicates normoxia just before CA. (D, E) Statistical analyses of cell viability during 10 min reperfusion and necrosis after 1 h of reperfusion. The treatment conditions are similar to those described for (C). Values are means ± SEM. $n = 6–8$ for (D) and $n = 8–11$ for (E). *$P < 0.05$ vs. Control. (F) Representative western blot images of sAC expression and statistical analysis of necrosis performed in H9C2 cells after 2 h CA and 1 h reperfusion. Cells were transfected either with sAC-targeted (shRNA) or scrambled (scr) shRNA. $n = 4–5$. *$P < 0.05$ vs. scr at CA/R.

during reperfusion with 30 µmol/l KH7, which significantly reduced cellular cAMP (Fig. 4A), prevented recovery of Ca^{2+} homeostasis and cell viability after CA (Fig. 4C-E), whereas KH7 at this concentration has no effect on viability of normoxic cells (Fig. 4B). Similarly to KH7, treatment with another sAC inhibitor, i.e., LRE1, augmented cell death during reperfusion (Supplementary Fig. 1D). To substantiate the role of sAC in reperfusion injury, sAC knockdown was performed in H9C2 cells. As demonstrated in Fig. 4F, sAC knockdown significantly elevated the number of necrotic cells after CA/R. Thus, in accordance with data obtained in cardiomyocytes, sAC activity during reperfusion plays an important role in survival of H9C2 cells.

To examine the importance of mitochondrial sAC during reperfusion, we overexpressed sAC in H9C2 cells in a mitochondria-targeted or non-targeted manner and tested their respective response to CA/R. Confocal microscopy analysis confirmed the predominant co-localization of mitochondria-targeted sAC with mitochondria (Supplementary Fig. 2C). Treatment of cells with CA/R led to marked cellular necrosis that was significantly reduced by overexpression of mitochondrial sAC (Fig. 5). Although expression of non-targeted sAC showed a protective effect, it was markedly less than that of the mitochondria-targeted sAC expression. Furthermore, in separated set of experiments we compared the protective effect of mitochondrial sAC overexpression against CA/R performed in bicarbonate-buffered or bicarbonate-free, HEPES-buffered medium. In line with data obtained in cardiomyocytes (Fig. 3), the absence of bicarbonate during CA/R prevented beneficial effect of mitochondrial sAC overexpression (Fig. 5C, D). Thus, mitochondrial sAC supports cell survival during CA/R.

3.3. Suppression of PDE2 during reperfusion improves mitochondrial cAMP homeostasis and cell viability

To further verify a beneficial role of the mitochondrial cAMP pool in the reperfusion-induced injury, we used a pharmacological approach to influence mitochondrial cAMP signaling in reperfused cells. Recent studies argue for PDE2 as a predominant intra-mitochondrial PDE isoform in various cell types, including cardiomyocytes [4,21]. Here we hypothesized that inhibition of PDE2 during reperfusion may increase mitochondrial cAMP level and, thus, support cell viability. To address this issue, the mitochondrial cAMP concentration was analyzed during CA/R in H9C2 cells transfected with mitochondria-targeted FRET-based cAMP sensor [8]. Applying this approach we observed no significant alteration in mitochondrial cAMP concentration during CA (Supplementary Fig. 3), whereas during reperfusion cAMP was first rapidly reduced and then slowly increased (Fig. 6A). Inhibition of PDE2 with Bay 60-7550 (Cayman Chemical) during reperfusion significantly increased mitochondrial cAMP level (Fig. 6B). Accordingly, inhibition of PDE2 significantly reduced cellular necrosis after CA/R (Fig. 6C). Of note, the beneficial effect of the PDE2 inhibition was abolished by depletion of bicarbonate in medium (Fig. 6C), i.e., by suppression of sAC activity.

In contrast to mitochondrial cAMP, the analysis of cytosolic cAMP

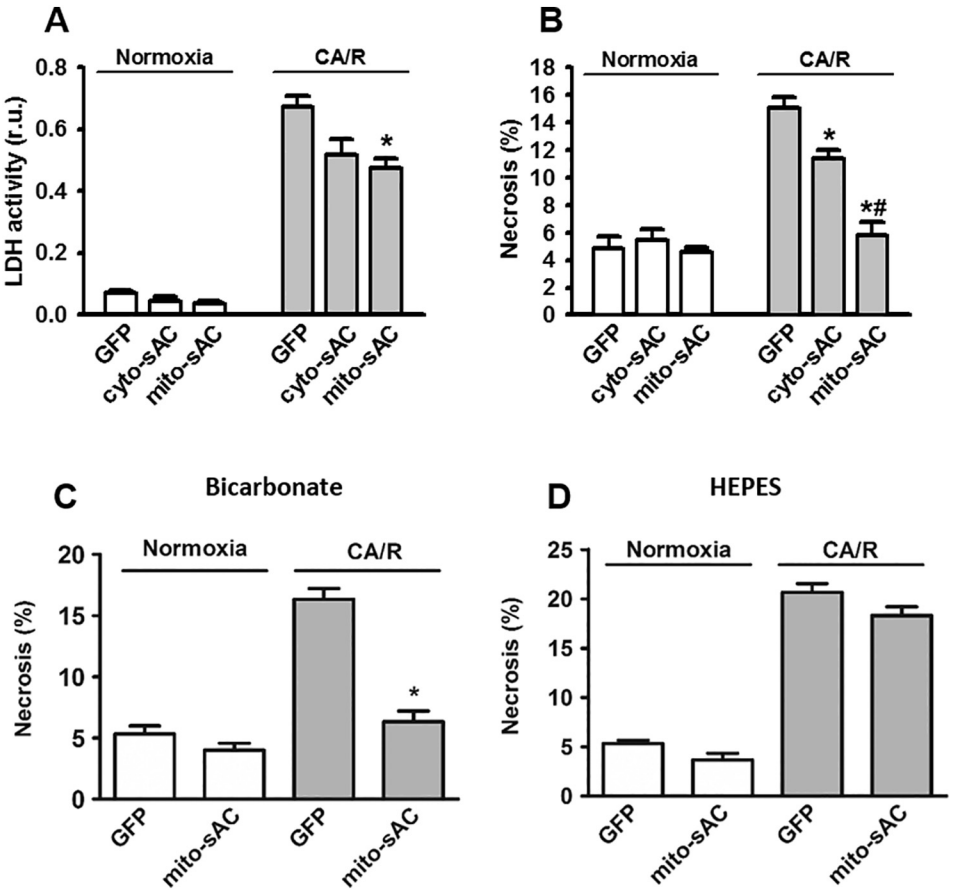

Fig. 5. sAC overexpression protects H9C2 cells against CA/R-induced injury. Statistical analysis of the lactate dehydrogenase (LDH) activity in the cell culture medium (presented as a ratio to individual protein content, relative units) (A) and necrosis (B) defined in cells after 2 h CA and 1 h reperfusion. Analyses were performed using cells transfected with a GFP-encoding vector, with a non-targeted (cyto-sAC) or mitochondria-targeted (mito-sAC) sAC-vector. Values are means ± SEM. $n = 3–5$. $^{*}P < 0.05$ vs. GFP at CA/R. $^{\#}P < 0.05$ vs. cyto-sAC at CA/R. (C–D) Statistical analysis of necrosis defined in H9C2 cells transfected with a GFP-encoding vector or mitochondria-targeted (mito-sAC) sAC-vector after 2 h CA and 1 h reperfusion. CA/R was performed either in bicarbonate-buffered (Bicarbonate) or in bicarbonate-free, HEPES-buffered (HEPES) medium. $n = 3$. $^{*}P < 0.05$ vs. GFP at CA/R.

level revealed no significant effect of PDE2 inhibition during reperfusion (Fig. 6B). Thus, inhibition of PDE2 during reperfusion affects the level of mitochondrial, but not cytosolic, cAMP.

To strengthen this finding we performed similar experiments in isolated adult rat cardiomyocytes exposed to CA/R. Bay 60-7550 treatment during reperfusion significantly improved cell viability, reduced necrosis, and accelerated the recovery of cytosolic Ca^{2+} homeostasis (Fig. 6D–F). Taken together, the data demonstrate a beneficial effect of PDE2 inhibition on mitochondrial cAMP content and cell viability during the acute reperfusion phase.

4. Discussion

The aim of the study was to examine the role of sAC in acute reperfusion-induced injury of cardiac cells. The main findings are as follows: (i) sAC is essential for cardiomyocyte survival during reperfusion; (ii) increasing mitochondrial sAC expression improves cell survival during reperfusion; (iii) inhibition of PDE2 during reperfusion increases mitochondrial, but not cytosolic, cAMP concentration and significantly improves cardiomyocyte survival.

To investigate the role of sAC in acute reperfusion injury, we simulated ischemia/reperfusion by applying metabolic inhibition with NaCN in glucose-free medium, i.e., chemical anoxia, followed by metabolic recovery. The similar model of simulated ischemia and reperfusion, i.e., anoxia/reoxygenation in isolated cardiomyocytes was characterized previously with respect to cytosolic ion homeostasis and cell morphology [11,12,18]. In contrast to an in vivo model, the in vitro model allows analysis of the injury mechanisms operating during reperfusion in cardiomyocytes independently from any effects of other cell types, e.g., endothelial or inflammatory cells. Furthermore, this model is free of excessive mechanical forces developing during reperfusion, i.e., hypercontracture, leading to cell death and effects of hemodynamics.

The model of chemical anoxia applied in the study is a widely used model to induce ATP depletion and seems to be appropriable to investigate the recovery of ATP synthesis after complete metabolic inhibition. Indeed, all characteristic features of anoxia/reoxygenation descried by our group previously [22–24] were reproduced with chemical anoxia, e.g., (i) rigor contracture within 20 min of chemical anoxia followed by rapid elevation of cytosolic Ca^{2+}; (ii) rapid initial fall of cytosolic Ca^{2+} followed by spontaneous Ca^{2+} oscillations during reperfusion; (iii) recovery of Ca^{2+} homeostasis within 15 min reperfusion.

Applying primary culture of adult rat cardiomyocytes subjected to CA/R we monitored cytosolic Ca^{2+} homeostasis and morphology. During CA the cardiomyocytes lose their energy reserves and, consequently, undergo rigor shortening. They develop a marked cytosolic overload of Ca^{2+} and H^{+}. The disturbance of ion homeostasis can be rapidly reversed when the cells are reperfused [25]. The ability of cells to rapidly restore cytosolic Ca^{2+} homeostasis plays a crucial role in cell fate during the initial phase of reperfusion. The recovery of cytosolic Ca^{2+} concentrations is strongly dependent on adequate energy support by mitochondria [23]. The irreversible loss of Ca^{2+} homeostasis observed in some cells in our study (Fig. 1C) was used as an indicator of ATP depletion and loss of cell viability.

Since the mitochondrial sAC-PKA axis promotes mitochondrial respiration and ATP synthesis under basal conditions [5], we hypothesized that mitochondrial sAC may beneficially affect cardiomyocyte viability during reperfusion. To test this hypothesis we suppressed sAC activity either pharmacologically with the specific sAC inhibitor KH7 or by bicarbonate withdrawal. Both approaches were found to significantly reduce the cellular cAMP content in control cardiomyocytes by about 50%. Similar reduction of cAMP, i.e., 30–50%, has been found in endothelial cell [15], neurons [13] and cancer cells [26]. In contrast, no significant alteration of cellular cAMP was observed under

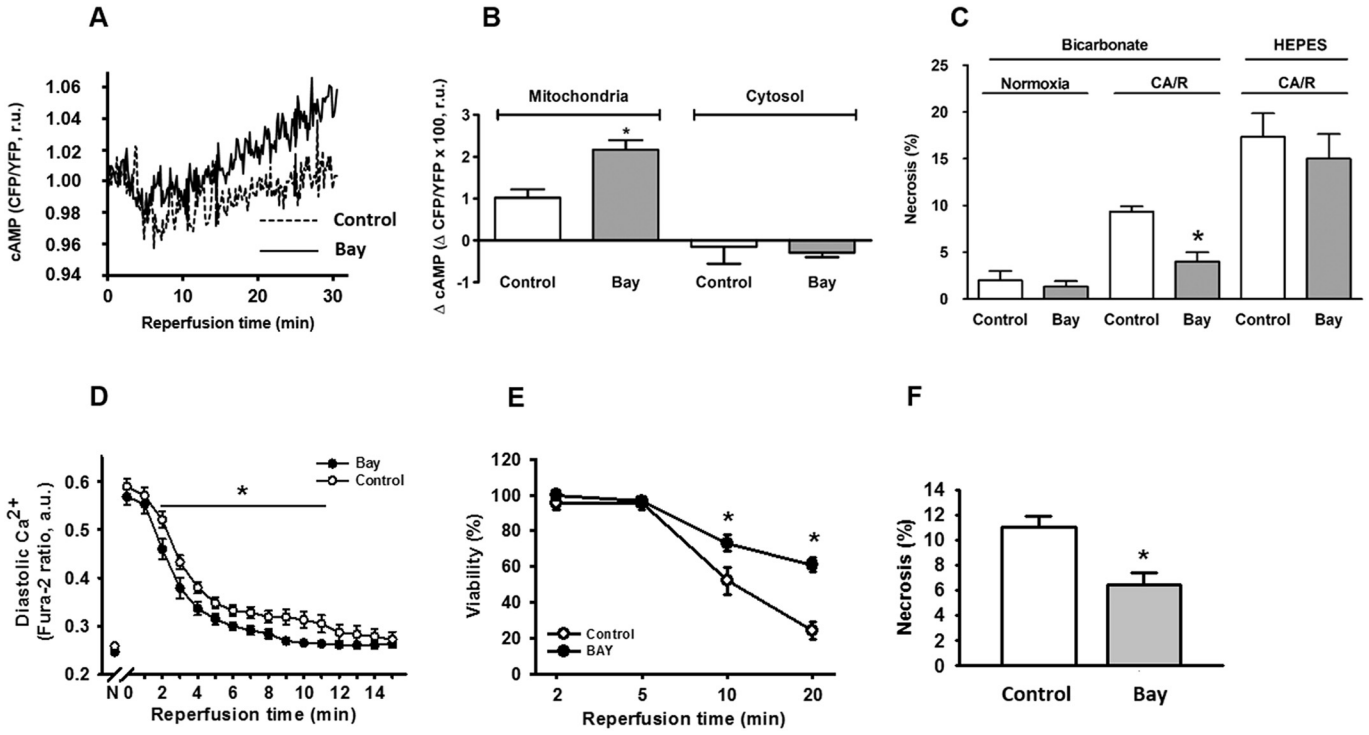

Fig. 6. Inhibition of PDE2 supports mitochondrial cAMP content and protects against reperfusion-induced injury. (A) Representative, original recordings of mitochondrial cAMP levels analyzed in H9C2 cells during reperfusion after 30 min of CA. Cells were treated during reperfusion either with 100 nmol/l Bay 60-7550 (Bay) or vehicle (Control). (B) Statistical analysis of the mitochondrial or cytosolic cAMP alteration in H9C2 cells during 20 min reperfusion. Values are means ± SEM. $n = 4–6$. *$P < 0.05$ vs. Control. (C) Statistical analysis of necrosis defined in H9C2 cells after 2 h normoxia or CA and 1 h reperfusion. CA/R was performed either in bicarbonate-buffered (Bicarbonate) or in bicarbonate-free, HEPES-buffered (HEPES) medium. Values are means ± SEM. $n = 3$. *$P < 0.05$ vs. Control at CA/R. (D, E) Time course of diastolic Ca^{2+} and cell viability observed in rat cardiomyocytes during reperfusion after 30 min of CA. Values are means ± SEM. $n = 5–6$. *$P < 0.05$ vs. control. (F) Statistical analysis of reperfusion-induced necrosis in cardiomyocytes during reperfusion after 30 min of CA. Values are means ± SEM. $n = 4–6$. *$P < 0.05$ vs. Control. The treatment conditions with Bay of vehicle (Control) for (B–F) are similar to those described for (A).

suppression of tmAC in cultured endothelial cells [15], though in other cell types, like INS-1E cells [27] or U937 cells [28], tmAC inhibition reduced the basal cAMP accumulation by 25–60%, which may reflect the presence of hormones in the medium.

In accordance with our hypothesis, sAC inhibition markedly reduces cell viability and recovery of Ca^{2+} homeostasis during reperfusion. Similarly to cardiomyocytes, sAC inhibition in H9C2 cells significantly disturbs Ca^{2+} homeostasis and cell viability during reperfusion. Thus, sAC activity is crucial for cardiomyocyte survival during reperfusion stress.

Though KH7 is a widely used, specific sAC inhibitor, recent reports demonstrated that it might have a side effect by depolarizing mitochondria [8,16]. Therefore, a novel sAC inhibitor, i.e., LRE1 [16], was also applied in cardiomyocytes and H9C2 cells during reperfusion. Similarly to KH7, LRE1 significantly reduced cell viability and augmented necrotic cell death in both cell types demonstrating reliability of the pharmacological sAC inhibition at reperfusion phase.

Our previous studies as well as reports of other groups suggest that extra- and intra-mitochondrial sAC may play an opposite role in cell viability. In particular, extra-mitochondrial sAC promotes the mitochondrial pathway of apoptosis [14,15,29], whereas intra-mitochondrial sAC supports mitochondrial function, resistance to Ca^{2+} overload, and attenuation of mitochondrial permeability transition pore opening [5,8,30,31]. Thus, to determine whether cytosolic or mitochondrial sAC beneficially affects cell viability during reperfusion, we overexpressed sAC in cardiac H9C2 cells in a mitochondria-targeted or -untargeted manner. Although the expression of sAC in extra- or intra-mitochondrial compartments significantly improves cell viability, the latter displays a significantly greater pro-survival effect. In agreement with our report, a recent study by Wang et al. [30] demonstrated a beneficial effect of intra-mitochondrial sAC for resistance of isolated mitochondria to Ca^{2+} stress. Interestingly, the authors found markedly lower sAC expression in cardiac mitochondria isolated from failing rat hearts than from control hearts, which was associated with reduced resistance of mitochondria to Ca^{2+} stress [30]. In addition, the seminal studies of Acin-Perez et al. demonstrated that cAMP produced in the mitochondrial matrix promotes cytochrome *c* oxidase activity via a PKA-dependent phosphorylation of cytochrome *c* oxidase subunit IV [4,5]. Similar results (regulation of OXPHOS activity via cAMP-PKA axis) were obtained in yeast, where the inhibition of sAC caused a decline in respiration and OXPHOS activity [104] suggesting an evolutionary conserved role of sAC in the regulation of mitochondrial function. In line with these studies, De Rasmo et al. [32] explained the beneficial role of mitochondrial cAMP in H9C2 cells via improved activity and coupling of mitochondrial ATP-synthase complex. Altogether, these previous studies and our findings argue for the beneficial effects of mitochondrial sAC via supporting mitochondrial function and encourage us to examine a clinically applicable approach to supporting mitochondrial cAMP signaling in reperfused cardiomyocytes.

Since the overexpression of mitochondrial sAC would be impractical in a clinical setting, we tested whether pharmacological suppression of mitochondrial cAMP degradation may support cell survival during reperfusion stress. To this aim a selective inhibitor of PDE2, Bay 60-7550, was applied. It has been shown that PDE2 is a major intra-mitochondrial isoform in liver and brain mitochondria [4], and its presence in cardiomyocyte mitochondria has also been confirmed [21]. Using a FRET-based probe during CA/R in H9C2 cells, we found no significant alterations in mitochondrial cAMP concentrations during CA, whereas during reperfusion a marked reduction in cAMP levels occurs that is followed by gradual recovery. PDE2 inhibition during reperfusion significantly accelerates cAMP recovery and improves survival of H9C2

cells and adult rat cardiomyocytes. Although we cannot exclude a possible effect of extra-mitochondrial PDE2 inhibition on cell viability, the analysis of cytosolic cAMP levels revealed no significant effect of PDE2 inhibition during reperfusion. Taken together, these data demonstrate a beneficial effect of PDE2 inhibition on cardiomyocyte viability during reperfusion. In agreement with this finding, a recent report suggested a protective effect of PDE2 inhibition in a brain ischemia/reperfusion model, although delayed rather than acute effects of reperfusion were analyzed in this study [33].

In conclusion, the current study argues for a beneficial role of sAC in cardiomyocyte viability during reperfusion stress. Furthermore, our results suggest a new pharmacological approach, i.e., PDE2 inhibition, to support mitochondrial cAMP signaling and cell survival in reperfused cardiomyocytes. It remains to be determined whether this approach can be extended to in vivo models and eventually translated to the clinical setting.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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Conflict of interest

The authors declare that they have no conflict of interest.

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