Role of soluble adenylyl cyclase in reperfusion-induced injury of cardiac cells

Ruolo della proteina adenilato ciclasi solubile nel danno indotto da riperfusione in cellule cardiache

Coordinatore:
Chiar.mo Prof. Riccardo Bonadonna

Tutori:
Chiar.mo Prof. Federico Quaini
Dr. Yaser Abdallah

Dottoranda:
Laura Rinaldi
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Summary

Aging- or stress-induced decline in mitochondrial function leads to a loss of cellular homeostasis and organismal health. Mitochondrial function is highly dependent on the expression and activity of proteins of the oxidative phosphorylation (OXPHOS) system. The mechanisms controlling activity of OXPHOS system are largely unknown. Recent evidence emphasized a role of mitochondrial cAMP/PKA signaling in promoting mitochondrial OXPHOS activity and ATP synthesis due to PKA-dependent phosphorylation of several subunits of the complex I and IV. It has been also shown that mitochondria possess an own source of cAMP, i.e., the type 10 soluble adenylyl cyclase (sAC). Ca²⁺ and bicarbonate promote sAC activity. Accordingly, in high-rate energy consuming tissues or organ, such as the heart, mitochondrial sAC activity might be particularly important to support the ATP synthesis in those pathological conditions where the energy homeostasis is disrupted, e.g., under ischemia/reperfusion (I/R). Therefore, in the present thesis the role of sAC in reperfusion-induced injury of cardiac cells was investigated. For this purpose, the pharmacological suppression of the sAC activity with specific inhibitor KH7, sAC stimulation with bicarbonate, and sAC overexpression were applied in adult rat cardiomyocytes and rat embryotic cardiomyoblasts (H9C2) subjected to simulated I/R. In both cell types reperfusion-induced injury, i.e., Ca²⁺ overload, hypercontracture, and necrotic cell death were analyzed. The data of this thesis demonstrate that inhibition of sAC activity in both cell types enhances reperfusion-induced injury. In contrast, supporting sAC activity with bicarbonate during ischemia and reperfusion or during reperfusion alone significantly preserves cardiomyocytes viability. Importantly, promoting sAC activity in mitochondria by targeted sAC overexpression in H9C2 cells markedly reduced reperfusion-induced injury.

In conclusion, during reperfusion the basal activity of sAC is important to support recovery of cardiomyocytes from ischemic insult. Stimulation of sAC during reperfusion further preserves cell viability. The data of the present thesis open perspectives for the development of new strategies effectively fighting post-ischemic cell death.
Riassunto

L’alterazione delle funzioni mitocondriali indotta da stress e invecchiamento comporta la perdita dell’omeostasi cellulare e della salute dell’organismo. La funzione mitocondriale è altamente dipendente dall’espressione e attività delle proteine appartenenti al sistema della fosforilazione ossidativa (OXPHOS). I meccanismi che controllano l’attività di tale sistema sono per lo più sconosciuti. Studi recenti mettono in evidenza un ruolo chiave del signaling mitocondriale di cAMP/PKA nel controllo dell’attività di OXPHOS grazie alla fosforilazione di diverse subunità del complesso I e IV. È stato dimostrato che i mitocondri posseggono una fonte propria di cAMP, ovvero l’enzima adenilato ciclasi solubile (sAC). Lo ione Ca$^{2+}$ e bicarbonato promuovono l’attivazione di sAC. Di conseguenza, in tessuti od organi ad alta richiesta di energia come il cuore, l’attivazione della sAC mitocondriale potrebbe essere particolarmente importante per promuovere la produzione di ATP in quelle condizioni patologiche in cui il normale rifornimento di energia è perturbato, come avviene durante ischemia/riperfusione (I/R). In questo progetto di tesi è stato studiato il ruolo di sAC nel danno indotto da riperfusione in cellule cardiache. A questo scopo, gli strumenti di soppressione farmacologica di sAC con l’inibitore specifico KH7, stimolazione di sAC con bicarbonato, e overespressione di sAC sono stati applicati in cardiomiociti di ratto adulto e cardiomioblasti di ratto embrionali (H9C2) sottoposti a esperimenti di simulata I/R. In entrambi i tipi cellulari, sono stati analizzati l’aumento della concentrazione di Ca$^{2+}$; l’ipercontrazione e la morte cellulare indotti da riperfusione. I risultati di questa tesi dimostrano che l’inibizione dell’attività di sAC in entrambi i tipi cellulari incrementa il danno indotto da riperfusione. Al contrario, il supporto dell’attività di sAC con bicarbonato, durante ischemia e riperfusione o durante la sola riperfusione, preserva significativamente la vitalità dei cardiomiociti. Inoltre, promuovendo l’attività di sAC nei mitocondri, tramite overespressione in cellule H9C2, viene considerevolmente ridotto il danno da riperfusione.

In conclusione, l’attività basale di sAC è importante durante la riperfusione per sostenere la ripresa dei cardiomiociti dall’insulto ischemico. La stimolazione di sAC durante la riperfusione preserva ulteriormente la vitalità cellulare. I risultati di questa tesi aprono prospettive per lo sviluppo di nuove strategie nella protezione del miocardio dalla morte cellulare derivata dall’insulto post-ischemico.
# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>2-DOG</td>
<td>2-deoxy-D-Glucose</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>ADCY10</td>
<td>Adenylate cyclase 10 (soluble)</td>
</tr>
<tr>
<td>AKAP</td>
<td>A-kinase anchoring protein</td>
</tr>
<tr>
<td>ARCs</td>
<td>Adult rat cardiomyocytes</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5′-triphosphate</td>
</tr>
<tr>
<td>Bad</td>
<td>BCL2-associated agonist of cell death</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>Bim</td>
<td>BCL-2-interacting mediator of cell death</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3′,5′-cyclic monophosphate</td>
</tr>
<tr>
<td>CA/R</td>
<td>Chemical anoxia-reperfusion injury</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis conductance regulator</td>
</tr>
<tr>
<td>cGMP</td>
<td>Guanosine 3′,5′-cyclic monophosphate</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>COX</td>
<td>IV-1 cytochrome oxidase</td>
</tr>
<tr>
<td>CVDs</td>
<td>Cardiovascular diseases</td>
</tr>
<tr>
<td>Cyt c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>cyto-sAC</td>
<td>Cytosolic soluble adenylyl cyclase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EPAC</td>
<td>Exchange protein activated by cAMP</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5′-triphosphate</td>
</tr>
<tr>
<td>HCO$_3^-$</td>
<td>Bicarbonate</td>
</tr>
<tr>
<td>H9C2</td>
<td>Embryonic cardiac myoblasts H9C2 cell line</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>IP$_3$R</td>
<td>Inositol 1,4,5-triphosphate receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>I/R</td>
<td>Ischemia-reperfusion injury</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>mito-sAC</td>
<td>Intra-mitochondrial soluble adenylyl cyclase</td>
</tr>
<tr>
<td>MPTP</td>
<td>Mitochondrial permeability transition’s pore</td>
</tr>
<tr>
<td>NaCN</td>
<td>Sodium cyanide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation system</td>
</tr>
<tr>
<td>PDE</td>
<td>Phosphodiesterase</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>RISK</td>
<td>Reperfusion injury salvage kinases</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarcoendoplasmic reticulum Ca(^{2+})-ATP-ase</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>sAC</td>
<td>soluble adenylyl cyclase</td>
</tr>
<tr>
<td>sAC(_{fl})</td>
<td>soluble adenylyl cyclase full length</td>
</tr>
<tr>
<td>sAC(_{tr})</td>
<td>soluble adenylyl cyclase truncated variant</td>
</tr>
<tr>
<td>tmAC</td>
<td>Transmembrane adenylyl cyclase</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TOM</td>
<td>Outer membrane translocase</td>
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</tbody>
</table>
1 Introduction

1.1 Cardiovascular diseases

Cardiovascular diseases (CVDs) are both common and deadly and they represent the leading cause of death worldwide. According to the WHO, 17.5 million people died from CVDs in 2012, representing 31% of all global deaths. It is estimated that this number will increase to 23.6 million by 2030. An unhealthy diet, sedentary lifestyle, smoking and the harmful use of alcohol represent behavioral risk factors for CVDs, and they may show up in individuals as raised blood pressure, raised blood glucose, raised blood lipids, overweight and obesity (http://www.who.int/cardiovascular_diseases/en/). Coronary heart disease (CHD) represents the most common CVD (13% of all deaths) and may lead to the myocardial ischemia culminating in myocardial infarction (Hausenloy DJ and Yellon DM, 2013; Chaturvedi N, 2003).

1.2 Myocardial ischemia and reperfusion-induced injury

Myocardial ischemia occurs when the proper coronary blood supply to the tissue is interrupted, resulting in the deprivation of oxygen and nutrients and the accumulation of metabolism’s products, such as lactic acid and CO2, inside the myocardium (Jennings and Steenbergen, 1985). Without treatment ischemia leads to cell dysfunction, injury and death: for this reason revascularization and restoration of the blood supply still represents the mainstay of all current therapeutic approaches to ischemia. Nevertheless, an additional damage is caused by the restoration of blood flow; this is known as reperfusion injury (Dirksen et al., 2007). Myocardial reperfusion has been postulated for the first time in 1960 by Jennings group which described the histological characteristics of reperfused ischemic myocardium in dogs. They reported cell swelling, myofibrils contraction, disruption of the sarcolemmal, and the appearance of intra-mitochondrial calcium phosphate particles (Jennings RB et al., 1960). Reperfusion injury refers to a causal event associated with reperfusion that had not occurred during the ischemic period and can be attenuated only by intervening at the time of reperfusion. This injury causes four type of cardiac dysfunction: myocardial stunning, no-reflow phenomenon, reperfusion arrhythmias and lethal reperfusion injury. Myocardial stunning denotes the “mechanical dysfunction that persists after
reperfusion despite the absence of irreversible damage and despite restoration of normal or near-normal coronary-flow” (Braunwald E and Kloner RA, 1982). Myocardial stunning disappears spontaneously, it becomes of serious concern only when ischemia affected a big part of the tissue. This is the reason why myocardial stunning is not a major clinical problem in the context of acute myocardial infarction. The no-reflow phenomenon refers to the inability of the microvascular blood flow to reperfuse an ischemic region (Ito H, 2006). Reperfusion arrhythmias is potentially dangerous but can be easily treated. Lethal reperfusion injury is defined as an injury caused by the restoration of blood flow after an ischemic event leading to death of those cells which are still alive or only reversibly injured at the end of the ischemic event. Whereas several comprehensive reviews of myocardial stunning, no-reflow phenomenon and reperfusion arrhythmias are available, the lethal reperfusion injury, as an independent mediator of cardiomyocyte death, has been debated for years and is still controversial. One of the main problems is the inability to accurately follow in time the development of necrosis during the transition from myocardial ischemia to reperfusion (Piper HM et al., 1998). Although is not possible to distinguish if cellular damage and death in a reperfused myocardium are due to reperfusion itself or entirely to the ischemic event, there are criteria for the presence of lethal reperfusion injury. These criteria are based on the observation of the infarct size reduction by intervention used only at the beginning of reperfusion.

Early studies in this area had shown that interventions during reperfusion aimed at controlling cytosolic calcium (Ca\(^{2+}\)) concentration or the activation of the contractile machinery in cardiomyocytes could reduce cardiac cell necrosis (Siegmund et al., 1991; Abdallah et al., 2006.). More recently, it has been found that a protective protocol called “post-conditioning” (Zaho ZQ et al., 2003) can have a clinical use by applying brief and repetitive bouts of ischemia during the first minutes of reperfusion. It is also becoming clear that the activation of specific protein kinases, named reperfusion injury salvage kinases (RISK) can mediate cardiac protection during the reperfusion period (Tsang A et al., 2004; Gateau-Roesch O et al., 2006).

Despite the reperfusion injury has been investigated for long time and by many researchers, we still do not have a definitive intervention to eliminate reperfusion-induced myocardial damage. Therefore, it is important to fully understand the mechanisms of ischemia-reperfusion injury and to consider cardioprotective strategies.
1.3 Ischemia

1.3.1 ATP depletion

To maintain cellular homeostasis, the intracellular use of ATP and high energy phosphates is critically important. Cardiomyocytes are rich in mitochondria since they are mainly dependent on the aerobic metabolism. The absence of oxygen stops the oxidative phosphorylation leading to the depolarization of mitochondrial membrane, ATP depletion, and inhibition of myocardial contractile function. ATP is consumed at a much faster rate than it is produced and its concentration drops to <2.0 μmol/g dry weight after an hour of severe ischemia versus the 25 to 27 μmol/g dry weight in a normal myocardium (Jennings RB, 2013). The low ATP is highly associated with cell death during the myocardial ischemia event (Jennings RB et al., 1990). ATP depletion is directly involved in cardiomyocytes contracture which is typical of the myocardial ischemic event, called rigor-contracture. In the ischemic myocardium, after cytosolic ATP is reduced to a low level (<100 μmol/l), a contraction is generated by slow cross-bridge cycling (Altschuld RA et al., 1985a; Nichols CG and Lederer WJ, 1990). This window of low ATP concentrations is opened only for a short period of time since cellular ATP reserves are quickly exhausted. Thus, the myofibrillar shortening remains fixed as all cross-bridges between actin and myosin stay in an attached state. Rigor-contracture, typical of myocardial ischemia, does not cause a severe structural damage but it impairs the cytoskeleton. The ischemic rigor contracture is found to be reversible if re-energization occurs. Reoxygenation within 5 minutes avoids irreversible cellular damage, whereas ischemia for more than 15 minutes gradually affects intracellular structures (Mauser M et al. 1985).

1.3.2 Disruption of cytosolic ion homeostasis and contributing mechanisms

During ischemia, oxidative phosphorylation rapidly ceases and anaerobic glycolysis is accelerated. Lactate and protons, generated by glycolysis, together with proton release due to ATP degradation result in cell acidosis of about 1 pH unit within 10 minutes (Neely JR and Grotyohann LW, 1984). Whereas the development of severe acidosis (tissue pH ≤6.6) aggravates tissue injury caused by oxygen and substrate deprivation (Nayler et al., 1979; Acosta and Li, 1980; Preusse et al., 1982), the effect of mild acidosis (6.6≤ pH ≤7.4), during the early stage of ischemia, has been shown to exert a protective effect by reducing the energy demand (Koop A and Piper HM, 1992). The lactate
production leads to osmotic cell stress and water accumulation in cells causing sarcolemmal disruption.

To buffer the intracellular acidosis, protons are excreted through different mechanisms, including Na\(^+\)/H\(^+\) exchanger and Na\(^+\)/HCO\(_3\)\(^-\) symporter. The action of these transporters causes a substantial Na\(^+\) influx (Pike MM et al. 1993; Sanada S et al., 2011). Under ATP depletion Na\(^+\) extrusion is impaired since the Na\(^+\)/K\(^+\)-ATPase of the sarcolemma is inhibited by the lack of ATP: the rise in Na\(^+\) concentration will be minimized by the activation of the Na\(^+\) pump at least at the beginning of ischemia. The increase in intracellular Na\(^+\) concentration affects the Na\(^+\)/Ca\(^{2+}\) exchanger, sensible to Na\(^+\) gradient. The Na\(^+\)/Ca\(^{2+}\) exchanger starts to operate in the reverse mode, extruding three Na\(^+\) ions and transporting one Ca\(^{2+}\) ion inside the cell. Consequently the ischemic cardiomyocyte develops cytosolic Ca\(^{2+}\) overload (Piper HM et al., 2003).

Accumulation of Ca\(^{2+}\) inside the cell is a common feature of injury and promotes cell damage by a range of mechanisms including activation of proteases and phospholipases, and possibly impairment of oxidative phosphorylation. In irreversibly injured cardiomyocytes this processes will eventually lead to the injury of membrane phospholipids and ion channels, and decrease of ATP production, ultimately accelerating cell necrosis.

During ischemia a large efflux of K\(^+\) occurs, leading to the depolarization of the membrane and making the cell unexcitable. This effect is protective, since the demand of ATP is reduced. K\(^+\) leaks rapidly from an ischemic cardiomyocyte, increasing its concentration in the extracellular fluid. K\(^+\) efflux in ischemia occurs mainly through K\(^+\) channels sensible to ATP, which has a higher open probability when ATP in the intracellular space is reduced (Noma A, 1983).

### 1.3.3 Ischemic mitochondrial injury

During myocardial ischemia, mitochondrial ultrastructural and functional injury also occurs in a time-dependent and progressive way (Lesnfsky EJ et al., 2001; Murry CE et al., 1990). In the first 10 minutes of ischemia mitochondrial ATP-synthase and nucleotide translocases activity is reduced (Rouslin W, 1983; Duan J and Karmazyn M, 1989), followed by the activity reduction of complex I of the electron transfer chain and mitochondrial depolarization. (Levrault J et al., 2003). These modifications are reversible if reperfusion occurs within these 30 minutes (Flameng W et al., 1991). Prolonged ischemia more likely results in irreversible cell impairment and is associated with
defects in complexes III and IV (Lesnefsky EJ et al., 1997) and reduction of the inner mitochondrial membrane potential. Furthermore, during ischemia ATP-synthase may operate in a reverse-mode leading to ATP hydrolysis to facilitate the maintenance of the proton-motive force. The higher rate of ATP hydrolysis accelerates the establishment of irreversible ischemic injury and mitochondrial dysfunction.

In the myocardial tissue, the electron transfer chain is the main source of reactive oxygen species (ROS). During ischemia ROS generation cannot occur unless some residual oxygen (O2) is still present. Studies using isolated cardiomyocytes suggested that O2 traces are still detectable during simulated ischemia (Po2 = 5-7 mm/Hg). Indexes of oxidative stress were attenuated by mitochondrial electron transport inhibitors, suggesting that ROS are generated by mitochondria (Becker LB et al., 1999; Vanden Hoek TL et al., 1997). A work by Levraut's group in 2003 demonstrates that mitochondria undergo a significant and irreversible decrease in potential during ischemia and the degree of depolarization correlates with the extent of cell death during reperfusion. Mitochondrial depolarization during ischemia has been suggested to be triggered by ROS generated by the mitochondrial electron transport chain despite the low concentration in O2. These oxidants initiate a cascade of lipid peroxidation that disrupts the integrity of the inner mitochondrial membrane leading to cell death by promoting matrix swelling, release of cytochrome c in the cytosol and apoptosis. Activation of the mitochondrial permeability transition’s pore (MPTP) apparently do not contribute to this process because cyclosporine A treatment has no effect on the decrease in membrane potential during ischemia or cell death (Levraut J et al., 2003). This is consistent with the evidence that MPTP opening is in part inhibited by the acidosis occurring during ischemia, whereas is more likely to occur after reperfusion (Halestrap AP et al., 1998; Kerr PM et al., 1999). Another main feature of cell injury is mitochondrial Ca2+ overload. Ischemia brings a slow and progressive increase in basal cytosolic Ca2+ concentration. This maintained increment gives rise to a proportionally greater increase in mitochondrial Ca2+ concentration until mitochondrial Ca2+ overload occurs (at about 1-3 µmol/l cytosolic Ca2+) (Nicholls DG, 1978). Accumulation of Ca2+ itself is probably innocuous, but in the presence of exogenous adenine nucleotides and high phosphates or H2O2, mitochondrial Ca2+ leads to MPTP opening. MPTP opening more likely happens within the first 5 minutes of reperfusion when more ROS are produced, the level of ATP is still low, and alkalization occurs.
1.4 Reperfusion

1.4.1 Re-energization and recovery of ion homeostasis

Reperfusion brings a rapid restoration of substrates essential for the generation of ATP, an instantaneous increase in the O_2 supply and normalization of the extracellular pH. When reperfusion occurs, if the mitochondria function to produce ATP has not been critically impaired by the previous ischemic period, reoxygenation leads to the recovery of oxidative energy production. Re-synthesis of ATP can reactivate the contractile machinery and the cation ATPases, such as Na^+/K^+-ATPase and sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA). SERCA can now hydrolyze ATP to clean the cytosol from Ca^{2+} overload, moving Ca^{2+} inside the sarcoplasmic reticulum (SR) (Siegmund B et al., 1992; Siegmund B et al., 1994). If the capacity of SR is too small for the amount of Ca^{2+} accumulated in the cytosol, the excess of Ca^{2+} is released in the cytosol through ryanodine receptors (RyR) and therefore, a cycle of continuous release and reuptake of Ca^{2+} from and into the SR takes place. During the early phase of reperfusion, the cytosolic Ca^{2+} levels are still very high and therefore myofibrillar activation leads to uncontrolled force generation. This sustained force generation causes hypercontraction, which is an irreversible structural damage (Siegmund B et al., 1997; Schäfer C et al., 2001) (Figure 1.1).

The Ca^{2+} oscillatory shifts lead to high cytosolic Ca^{2+} concentrations. The frequency of these Ca^{2+} peaks is influenced by an ongoing Ca^{2+} influx across the sarcolemma during the early phase of reperfusion (Schlüter KD et al., 1996). These oscillations cease only if the major mechanism for Ca^{2+} extrusion, i.e. the Na^+/Ca^{2+} exchanger of the sarcolemma, is sufficiently activated in its “forward mode”. During ischemia the exchanger is operating by removing Na^+ from the cytosol and uptaking Ca^{2+}, in its “reverse mode” (Piper HM et al., 1998). To activate the “forward mode” of Na^+/Ca^{2+} exchanger, the restoration of Na^+ gradient across the sarcolemma is a prerequisite for extrusion of Ca^{2+} from cardiomyocytes (Piper HM et al., 1998). With reactivation of oxidative phosphorylation, the Na^+/K^+-ATPase is reactivated generating a trans-sarcolemmal Na^+ gradient, which provides driving force for the Na^+/Ca^{2+} exchanger in its “forward mode”. It seems that cells in which these pumps have been crucially damaged during the ischemic period are unable to recover and cannot be subjected to reperfusion injury described above (Piper HM et al., 1998).

Upon reperfusion both extracellular and intracellular pH are quickly renormalized and an extreme H^+ gradient is generated between the cytosol, containing still high Ca^{2+} concentration, and the
extracellular space across the plasma membrane. The alkalization of extracellular space leads to the activation of the H⁺ extruding mechanisms: the Na⁺/H⁺ exchanger and the Na⁺/HCO₃⁻ symporter (Lagadic-Gossmann D and Vaughan-Jones RD, 1993; Piper HM et al., 1996). The extrusion of H⁺ from the cell removes a potentially protective agent (acidosis) and the activation of Na⁺/H⁺ exchanger causes a massive Na⁺ influx, which in turn may trigger a secondary and temporary activation of the Na⁺/Ca²⁺ “reverse mode” exchange, enhancing the pre-existing cytosolic Ca²⁺ overload.

### 1.4.2 Mechanisms of reperfusion-induced cardiomyocyte injury

Several mechanisms are involved in cardiomyocyte injury upon reperfusion, including restoration of normal pH, oxidative stress, Ca²⁺ oscillations, and hypercontraction. Experimental studies have demonstrated that the reperfusion of ischemic tissue generates oxidative stress which can mediate myocytes injury (Zweier JL, 1988). Oxidative stress reduces the bioavailability of the intracellular signaling molecule, nitric oxide (NO), by removing its protective effects, including inhibition of neutrophil accumulation, inactivation of superoxide radicals, and improvement of blood flow (Zweier JL and Talukder MA, 2006).

The rapid re-alkalization and re-energization of the cell together with Ca²⁺ oscillations between SR and cytosol immediately trigger an uncontrolled and excessive activation of myofibrils. This sustained force generation causes an irreversible cell shortening state called hypercontraction. In tissue, the communication through gap junctions between adjacent cells may lead to the spread of cell injury during myocardial infarction (García-Dorado D et al., 1989). The passage of Na⁺ from the hypercontracting cell to the adjacent cell and the subsequent activation of the Na⁺/Ca²⁺ “reverse mode” exchange may result in the propagation of contracture (Ruiz-Meana M et al., 1999). Reperfusion-induced hypercontraction observed in reperfused myocardium after prolonged ischemia is characterized by greater myofibrillar shortening, cytoskeletal damage, and sarcolemmal disruption if compared to myocardium in ischemic rigor contracture. This histological picture is known as “contraction band necrosis” (Ganote CE, 1983).

It has been shown in vivo that continuation of acidosis during the early phase of reoxygenation protects cardiomyocytes against the development of hypercontraction and necrosis. This protection might be achieved in vivo by the inhibition of Na⁺/H⁺ exchanger applied during the
ischemic period (Bugge E and Ytrehus K, 1995; García-Dorado D et al., 1997). However, in clinical studies, delaying the restoration of physiologic pH during myocardial reperfusion through Na+/H+ exchanger inhibition did not protect the heart (Zeymer U et al., 2001; Avkiran M and Marber MS, 2002).

During this sequence of events, Ca2+ overload and ROS generation can trigger multiple modes of cell death, among which necrosis and apoptosis are the most common (Sanada S and Kitakaze M, 2004). Necrosis prevails within the ischemic myocardium and its adjacent regions, whereas apoptosis predominantly occurs in the ischemic border and in non-ischemic regions (Takemura G and Fujiwara H, 2006). Myocardial necrotic process is mainly caused by ATP depletion, cytosolic Ca2+ rise followed by activation of phospholipases and proteases, and cellular swelling due to cytosolic Na+ overload. The Na+/H+ exchanger plays a major role in cell volume regulation (Inserte J et al., 1997; Grinstein S et al., 1992). Upon reperfusion an osmotic gradient between the extracellular and the intracellular space is generated leading to cellular uptake of water and, through the consecutive increase in intracellular pressure, mechanical stretch of sarcolemma, which might end in the rupture of cellular membrane (Armstrong SC and Ganote CE, 1992; Ruiz-Meana M et al., 1996). Cellular swelling and cell membrane rupture might be followed by other necrotic changes, such as degradation of intracellular proteins or structures induced by Ca2+-dependent proteases (e.g. calpain), hypercontraction and direct cleavage of DNA by free radicals. Changes induced by necrosis generally require focal recruitment of inflammatory cells for the subsequent scavenging activity.

1.4.3 Mechanisms of reperfusion-induced mitochondrial injury

The restoration of blood flow restores oxygen and substrate supply and removes residual metabolites harmful for mitochondrial and myocytes recovery. Nevertheless, if ischemic damage to complexes III and IV is significant, the recovery of energy is limited and reperfusion exacerbates cell injury (Veitch K et al., 1991). At the time of reperfusion, when O2 is reintroduced into the cell, the production of superoxide (O2-) increases, both outside mitochondria and in the mitochondrial electron transport chain (Morin D et al., 2001). In this setting, duration and severity of the previous ischemic event are important to determine the extent of reperfusion injury (Kirshenbaum LA and Singal PK, 1992; Murphy et al., 1984). Under normal conditions, mitochondria have an antioxidant system based on two enzymes, the superoxide dismutase (SOD) and glutathione peroxidase (GPX),
able to neutralize the ROS species $O_2^-$ and hydrogen peroxide ($H_2O_2$), respectively. Conversely, during reperfusion these antioxidant defenses are weakened; consequently, SOD deficiency can result in high levels of peroxynitrite and low levels of reduced glutathione, the GPX substrate, associated with the increased production of hydroxyl radical (OH). Mitochondrial ROS generation may also be stimulated by the elevated pro-inflammatory cytokines levels during reperfusion injury (García-Ruiz C et al., 1997). The increase in ROS production and a reduced antioxidant capacity result in three main types of mitochondrial damage. The first type of injury is represented by proteins oxidation, particularly in the respiratory chain, which leads to structural alterations and inhibition of mitochondrial respiration. The second type of injury consists in the alteration of lipid metabolism; in this setting, lipoperoxidation occurs and generates products dangerous for the integrity of mitochondrial membranes, by increasing the permeability of the inner membrane (Chen JJ and Yu BP, 1994) and altering its fluidity, which in turn leads to increased $H_2O_2$ production (Chen X and Gross RW, 1994). Finally, pyridine nucleotides oxidation may occur, leading to membrane permeability changes, oxidative phosphorylation uncoupling, and ATP production collapse (Costantini P et al., 1996; Nieminen AL et al., 1997).

In response to myocardial ischemia and reperfusion, $Ca^{2+}$ influx in the mitochondrial matrix is facilitated, in part by ROS mediated calcium release from the sarcoplasmic reticulum (Duchen MR, 2004). An excessive $Ca^{2+}$ influx into the mitochondria can contribute to the increase in permeability of the inner mitochondrial membrane, inhibition of oxidative phosphorylation (Crompton M, 1999), and enhancement of ROS generation (Brookes PS et al., 2004).

The MPTP embodies the pathological effects of $Ca^{2+}$ on mitochondria. The MPTP is an assembly of preexisting proteins of the inner and outer mitochondrial membranes into a large conductance channel permeable to molecules with a mass of less than 1500 Da. The MPTP is triggered by high mitochondrial $Ca^{2+}$ concentrations, oxidants, and the depletion of adenine nucleotides. During ischemia-reperfusion injury, particularly during reperfusion, mitochondria increase the uptake of $Ca^{2+}$, functioning as a sequestering system, in order to maintain the stability of cytosolic $Ca^{2+}$ concentration. There is evidence that the combination of high $Ca^{2+}$ concentrations inside mitochondria and enhanced ROS production leads to MPTPs opening, which in turn causes the dissipation of the mitochondrial membrane potential and the release of cytochrome $c$ from mitochondria to the cytosol, giving initiation to the apoptosis pathway (Skulachev VP, 2000; Frey
TG and Mannella CA, 2000) (Figure 1.1). The release of pro-apoptotic factors which are normally confined to the interior of mitochondria triggers a sequence of events that starts with the binding of cytochrome c to apoptosis protease-activating factor 1 (Yang J et al., 1997; Kluck RM et al., 1997). In the presence of ATP, this complex activates the apoptosis pathway through the cleavage of procaspase 9 to caspase 9, followed by activation of the downstream caspase 3, and apoptosis. In addition, it has been suggested that cytochrome c can bind to the endoplasmic reticulum (ER) inositol 1,4,5-triphosphate receptor (IP₃R), resulting in enhanced Ca²⁺ release (Boehning D et al., 2003; Boehning D et al., 2004). Thus, Ca²⁺-induced mitochondrial cytochrome c release may propagate apoptotic signaling by promoting further Ca²⁺ overload. Taken together this data demonstrate that mitochondria control biological responses including the cell ability to respond to pathophysiological insults such as ischemia and reperfusion injury.

**Figure 1.1 Scheme of pathogenesis of acute reperfusion injury.** Reperfusion reactivates ATP production in mitochondria (Mito). Recovering energy production (High ATP) activates SERCA on the sarcoplasmic reticulum (SR), which clears the cytosol from Ca²⁺ overload accumulated during ischemia. Repetitive release of Ca²⁺ through RyR and reuptake into the SR leads to Ca²⁺ oscillations with high cytosolic Ca²⁺ concentrations. High Ca²⁺ together with ATP triggers myofibrillar hypercontracture (Ca²⁺ contracture) and subsequent disruption of cells (Necrosis). Ca²⁺ uptake through the uniporter into mitochondria causes the opening of MPTP and Cyt c release, leading to failure of energy production (low ATP) and activation of apoptosis. Low ATP induces rigor contracture of the myofibrils, again leading to cell disruption. Protection by RISK may interfere favourably at the SR or at mitochondria (Piper HM et al., 2006)
1.5 Mitochondrial cAMP signaling and myocardial ischemia

1.5.1 cAMP signaling

Adenosine 3’, 5’-cyclic monophosphate (cAMP) is a ubiquitous second messenger playing critical roles in diverse signaling pathways in all cell types. Described for the first time in 1958 by Sutherland and Rall, it was found to mediate hormone action in mammalian heart and liver (Sutherland EW and Rall TW, 1958; Rall TW and Sutherland EW, 1958). Within little more than a decade from its discovery, cAMP was shown to mediate the action of multiple hormones, neurotransmitters and growth factors, consequently regulating a wide range of cellular processes, including proliferation, differentiation, secretion, apoptosis, adhesion, and migration (Beavo JA et al., 1975).

cAMP is universally generated by a class of enzymes named adenylyl cyclase (AC) which catalyze the conversion of adenosine triphosphate (ATP) to cAMP and pyrophosphate. Six distinct subclasses of ACs have been identified, all catalyzing the same reaction but representing unrelated gene families. Class III is the most extensively studied since it includes the ACs expressed in the human genome, although they have been found also in some bacteria. Two types of ACs are present in mammals, transmembrane AC (tmAC) and soluble AC (sAC). As their name indicates, tmACs have a transmembrane domain and are regulated by G proteins (Taussig R and Gilman AG, 1995).

In mammals, cAMP has four main effectors: protein kinase A (PKA), exchange protein activated by cAMP (EPAC), cyclic nucleotide regulated ion channels (Kamenetsky M et al., 2006; Sunahara RK and Taussig R, 2002; Kopperud R et al., 2003), and some phosphodiesterase (PDE) (Gross-Langenhoff M et al., 2006).

**Protein kinase A (PKA).** PKA is the major downstream effector of cAMP signaling. It is composed of two catalytic subunits and two regulatory subunits, forming a heterotetramer in its inactive form (Beavo JA et al., 1975). PKA shows two binding domains for cAMP on each regulatory subunit. Upon binding of a total number of four cAMP molecules on the regulatory subunits, the inactive form of the protein is dissociated into one dimer of regulatory subunits and two active catalytic subunits, which represents the active form of PKA. Activated PKA phosphorylates and activates cytosolic and nuclear targets at serine-threonine, including ion channels (Anderson MP et al., 1991; Catterall WA, 2015; Stival C et al., 2015), cellular motor proteins (Scherer J et al., 2014; Kashina AS et al., 2004), transcriptional factors, and many enzymes (Dhillon et al., 2002).
The regulatory subunit dimer of PKA is important for the localization of the protein inside the cell through the bond to A-kinase anchoring protein (AKAP). AKAPs bind many other signaling proteins, creating a very efficient signaling hub at specific subcellular structures (Wong W and Scott JD, 2004; Scott JD et al., 2013). The compartmentalization of signaling proteins promotes the efficiency of cAMP signaling transduction and in addition allows the second messenger cAMP to mediate distinct physiological responses.

**Exchange protein activated by cAMP (EPAC).** After cAMP and PKA discovery in 1957 and 1968 respectively, two different research groups discovered in 1998 two distinct genes encoding for the proteins EPAC1 and EPAC2 (de Rooij et al., 1998; Kawasaki et al., 1998). EPAC1 and 2 are structurally very similar and contain a cAMP-binding domain homologous to that of PKA regulatory subunits. EPAC proteins bind to cAMP with high affinity and activate the Ras superfamily small GTPases Rap1 and Rap2. Many cAMP-mediated effects that were previously thought to act through PKA alone may also be triggered by EPAC. Extensive studies have established that EPAC proteins are involved in many of cAMP-related cellular functions such as cell adhesion (Rangarajan S et al., 2003; Enserink JM et al., 2004), cell-cell junction (Cullere X et al., 2005; Kooistra MR et al., 2005), exocytosis/tetretion (Ozaki N et al., 2000; Li J et al., 2007; Seino S and Shibasaki T, 2005; Maillet M et al., 2003), cell differentiation (Kiermayer S et al., 2005) and proliferation, gene expression, apoptosis, cardiac hypertrophy, and phagocytosis (Takai Y et al., 2001; Mitin N et al., 2005; Wennerberg K et al., 2005).

**Phosphodiesterase (PDE).** PDEs are the unique enzymes able to deactivate cAMP and in turn its pathway (Francis SH et al., 2001; Maurice DH et al., 2003; Manganiello VC and Degerman E, 1999), by catabolizing cAMP into 5'-AMP. PDEs are therefore important regulators of signal transduction mediated by this second messenger. The PDE family includes twelve members (PDE1-12) which are functionally related, although they may differ in their regulatory and kinetic properties. PDE members are distinguished for their substrate specificity, mechanism of action and subcellular location. Some PDEs are cAMP-selective hydrolases (PDE4, 7 and 8); others are cGMP-selective (PDE5, 6, and 9). Another group of five PDEs can hydrolyse both cAMP and cGMP (PDE1, 2, 3, 10, and 11). PDE12 cleaves cAMP and oligoadenylylates. PDEs may be activated by Ca$^{2+}$/calmodulin (PDE1), cGMP (PDE2), or cAMP (PDE10) (Conti M and Jin SL, 1999; Beavo JA, 1995; Verde I et al., 1999).
1.5.2 cAMP signaling in the mitochondrial matrix

cAMP produced by tmA
C and sAC can freely diffuse through the cytosol and can activate PKAs on the surface of the outer mitochondrial membrane. Furthermore, as the outer membrane of mitochondria is permeable to molecules smaller than 5 kDa (Zalman LS et al., 1980), cAMP might also act inside the inter-membrane space.

The activation of PKA by cytosolic cAMP can lead to the phosphorylation of three components of the outer membrane translocase (TOM) slowing down the import of mitochondrial proteins and promoting the switch from the aerobic to the anaerobic metabolism in conditions of high glucose or reduced oxygen availability (Schmidt O et al., 2011; Gerbeth C et al., 2013; Rao S et al., 2012). Increased cytosolic cAMP in response to bioenergetics stresses can also affect mitochondrial fission through the action of PKA: the phosphorylation of the dynamin-related protein (Drp1) prevents its translocation to the mitochondrial surface, leading to mitochondrial elongation rather than fission, which promotes cell survival (Gomes LC et al., 2011; Cribbs JT and Strack S, 2007; Chang CR and Blackstone C, 2007; Merrill RA et al., 2011). Moreover, several apoptosis-related proteins are substrates of PKA including the pro-apoptotic proteins Bax (Kumar S et al., 2009; Appukuttan A et al., 2012), Bad (Harada H et al., 1999; Danial NN et al., 2003; Yang J et al., 2010; Bui M et al., 2010), and Bim (Moujalled D et al., 2011). In particular PKA phosphorylation of Bax promotes its translocation to mitochondria triggering cytochrome c release and apoptosis (Kumar S et al., 2009; Appukuttan A et al., 2012).

Conversely to the outer mitochondrial membrane, the inner mitochondrial membrane is impermeable even to small molecules since it lacks the presence of porins (Lindén M et al., 1984) and owns a high content of cardiolipin (Ferreira FM et al., 2003). Despite this, cAMP was found in the mitochondrial matrix of animals (Di Pilato LM et al., 2004) suggesting the existence of an intra-mitochondrial cAMP signaling. Mitochondria originate from bacteria, where cAMP regulates motility, metabolism, and DNA replication (Pesavento C and Hengge R, 2009). It might be logical that a local cAMP signaling have been retained in the mitochondrial matrix during evolution.

Although cAMP cannot freely diffuse across the inner mitochondrial membrane from the cytosol, its presence was found in mammalian mitochondria. Recent works from two different groups on human cell lines and primary rat cardiomyocytes have demonstrated that the mitochondrial inner membrane is impermeable to cAMP (Di Benedetto G et al., 2013; Lefkimmiatis K et al., 2013). The
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proposed model for the generation of cAMP involves a mitochondrial sAC that can synthesize cAMP locally in response to CO₂/HCO₃⁻ produced during the tricarboxylic acid (TCA) cycle (Acin-Perez et al., 2009b; Di Benedetto G et al., 2013; Lefkimmiatis K et al., 2013). Another study by Zhang’s group in 2015 has showed the existence of cAMP in the mitochondrial matrix of Drosophila cultured cells. Although the sources of cAMP may differ in the fly, since it lacks the sAC gene, the presence of cAMP in the mitochondrial matrix appears universal. The same group has shown, through a bimolecular fluorescence complementation assay, that PKA locates in the mitochondrial matrix (Zhang F et al., 2015), giving strength to the hypothesis of a localized cAMP/PKA signaling inside mitochondria. PKA seems to be the main effector of cAMP signaling in the mitochondrial matrix; this is supported by the fact that the 85% of PKA activity in purified mitochondria derives from the matrix fraction (Agnes RS et al., 2010). Furthermore, many enzymes in the TCA cycle and electron transport chain complex subunits are phosphorylated at the PKA action site (Zaho X et al., 2011; Grimsrud PA et al., 2012). Besides PKA, also PDEs have been found in the matrix where they can be inhibited by hydrogen sulfide in response to environmental stresses like hypoxia (Módis K et al., 2013). Thus, almost a complete set of cAMP signaling effectors have been found in the matrix supporting the existence of a localized cAMP pathway which may have crucial roles in both physiological and pathological conditions.

1.6 Soluble adenylyl cyclase (sAC)

According to the old paradigm tmACs was the unique source of cAMP in mammals. This model of cAMP signaling requires the activity of the AC at the membrane site and in turn the diffusion of cAMP through the cytosol to its targets, such as nucleus, mitochondria, and centrioles. The cAMP diffusion to reach distal targets may lead to the activation of more proximal located targets, like at the plasmalemmal site. Therefore, this model itself with the simple cAMP diffusion would diminish the specificity of the cAMP signal, considering also the presence of PDEs which deactivate cAMP preventing its diffusion. In 1977 the soluble isoform of AC was discovered in rat testis by Braun’s group (Braun T et al., 1977). More intense studies started in 1999 with the first isolation of sAC cDNA (Buck J et al., 1999). Conversely to tmAC, sAC is localized throughout the cell and in specific micro-domains, including nucleus, mitochondria, microtubules, and centrioles (Zippin JH et al., 2003; Zippin LH et al., 2004; Acin-Perez et al., 2009b). Subcellular localization of sAC, through the
interplay with PDE, enables the generation of cAMP in close proximity to its intracellular targets providing specificity and selectivity of action.

1.6.1 Genomic organization and biochemistry of sAC
Adenylyl cyclase (AC) enzymes belong to the nucleotidyl cyclase family, which comprises six different classes. The AC from *E. coli* and other AC enzymes from related gram-negative prokaryotes belong to Class I, ACs from pathogens, which are secreted and act like toxins, belong to Class II, and eukaryotic ACs belong to Class III. In mammals there are ten genes encoding for AC proteins (ADCY1-ADCY10). ADCY1-9 encode for the extensively studied subfamily of the tmACs, while the ADCY10 gene encodes for the ACs. ADCY10 is the only source for sAC in the cell and it includes 33 exons. Alternative splicing has been reported for sAC mRNA and a 187 kDa full-length enzyme (sAC₉) and a 50 kDa truncated variant (sAC₈) were described (Buck J et al., 1999). sAC₈ consists of two heterologous catalytic domains (C1 and C2), one autoinhibitory region (Chaloupka JA et al., 2006), a canonical P-loop, and a leucine zipper-like sequence (Buck J et al., 1999). sAC₉ consists only of the two catalytic domains (C1 and C2) and it has a cAMP-forming activity much higher (20-fold) than the full-length type (Wuttke MS et al., 2001). A third isoform of sAC (sAC_somatic) of 80-85 kDa, was identified in several human tissue and cell lines; sAC_somatic lacks of the C1 domain and it would require a partner protein for its activity (Kamenetsky M et al., 2006; Geng W et al., 2005). No one of these isoforms includes a transmembrane domain.

sAC activity requires two divalent cations in the catalytic site to coordinate binding and cyclizing of ATP. Mammalian sAC shows a low ATP affinity in presence of Mg²⁺ which is two orders of magnitude lower than observed for other ACs. The low substrate affinity of sAC appears relevant considering the fact that it enables the activation by intracellular Ca²⁺ which has been shown to increase sAC substrate affinity (Jaiswal BS and Conti M, 2003; Litvin TN et al., 2003). Furthermore, sAC shows an increase in activity in presence of bicarbonate (HCO₃⁻), whose source might be either metabolically generated or external of the cell (Cann MJ et al., 2003; Steegborn C et al., 2005) (Figure 1.2); this makes sAC the unique intracellular enzyme known to be directly activated by HCO₃⁻. A recent work by Zippin’s group demonstrated that sAC-generated cAMP in β-cells reflects alterations in intracellular ATP, suggesting that sAC is also required to regulate ATP levels (Zippin JH et al., 2013). Finally, a heme domain was discovered in sAC which may serve as NO or carbon
monoxide sensor, providing a possible link between NO/CO and cAMP signaling (Middelhaufe S et al., 2012).

**Figure 1.** Activation of sAC by HCO$_3^-$ and Ca$^{2+}$. Cytosolic sAC can be activated by HCO$_3^-$ derived from carbonic anhydrase (CA)-dependent hydration of (a) external and (b) metabolic CO$_2$; and/or (c) HCO$_3^-$ that enters via membrane transporters (e.g. anion exchanger, Na$^+$/HCO$_3^-$ symporter and CFTRs). sAC can also be activated by (d) Ca$^{2+}$ entering the cell via membrane transporters (e.g. voltage-dependent Ca$^{2+}$ channels and RyRs). (e, f) HCO$_3^-$ and Ca$^{2+}$ have been shown to potentially activate sAC in the nucleus and mitochondria (Tresguerres M et al., 2011).

### 1.6.2 Physiological roles of sAC

sAC has been found to play crucial roles in regulating cell signaling in several cell types of different organs including testis, kidney, eye, lung, pancreas, and brain. In mature sperm, sAC is the sole producer of cAMP and it plays an important role in the capacitation process resulting in sperm motility by activating asymmetrical flagellar beat and in the ability to perforate the egg's zona pellucida (Esposito G et al., 2004; Hess KC et al., 2005). sAC presence was found in the ascending loop of Henle in the kidney where is suggested to regulate both Na$^+$/K$^+$-ATPase and Na$^+$/K$^+$/2Cl$^-$.
cotransporters (Pastor-Soler N et al., 2003; Hallows KR et al., 2009). It was also demonstrated that sAC is co-localized in acid-secreting cells with V-type H+-ATPase regulating apical H+ secretion in the epididymis lumen (Paunescu TG et al., 2008). A subsequent report suggested that sAC can also increase cystic fibrosis conductance regulator (CFTR) activity, thereby regulating the secretion of airway surface liquid and mucus from airway glands (Wang Y et al., 2005; Baudouin-Legros M et al., 2008). The same result was obtained with experiments on bovine corneal endothelial cells, where sAC activation increased CFTR dependent secretion of Cl-, HCO3-, and ATP (Sun XC et al., 2004). The pharmacological inhibition with the specific inhibitor KH7 or siRNA downregulation of sAC significantly abolished the HCO3-induced stimulation of fluid secretion in cholangiocytes (Strazzabosco M et al., 2009). Another study revealed the involvement of sAC in NaCl and water absorption in toadfish intestine (Tresguerres M et al., 2010), and in Cl- and K+ secretions in the distal colonic epithelium of guinea pigs (Halm ST et al., 2010). sAC dependent growth processes have also been shown in neurons (cultured dorsal root ganglion and spinal commissural neurons), where the inhibition of sAC results in the arrest of netrin-1 induced growth cone elaboration and axonal growth; both responses where mimicked by sAC overexpression (Wu KY et al., 2006). Moreover, other studies have demonstrated that sAC is essential in neutrophils for the release of H2O2 induced by tumor necrosis factor (Han H et al., 2005). sAC seems to be physiologically involved also in bone formation and reabsorption (Geng W et al., 2009).

**1.6.3 Mitochondrial sAC in ischemia and reperfusion injury**

Recent studies by Ladilov's group demonstrated that different pro-apoptotic stimuli lead to the sAC translocation to mitochondria promoting the mitochondrial pathway of apoptosis via PKA-dependent Bax binding under simulated-ischemia/reperfusion injury (Kumar S et al., 2009; Appukuttan A et al., 2012). Thus, cytosolic sAC may under certain stress conditions promote apoptotic cell death.

Reports from other groups suggested that the sAC is also localized in the mitochondrial matrix (Acín-Perez R et al., 2009a-b; Di Benedetto G et al., 2013). Recent evidence emphasized a key role of mitochondrial sAC/cAMP/PKA signaling in controlling mitochondrial OXPHOS activity, ATP synthesis and attenuating the oxygen radical production (Acín-Perez R et al., 2009a-b; Di Benedetto G et al., 2013) (Figure 1.3). In particular, a sAC-dependent PKA activation in the matrix is suggested
to trigger the phosphorylation of the subunit IV-I cytochrome oxidase (COX) and likely of other enzymes of the respiratory chain. In accordance, the transport of bicarbonate into coronary endothelial cells during ischemia suppresses mitochondrial apoptosis (Kumar S et al., 2011). In this setting, the mitochondrial bicarbonate influx may lead to the activation of sAC which promotes the activity of the cytochrome C oxidase through the cAMP/PKA pathway, as suggested in a work by Acin-Perez et al. in 2009. They demonstrated that the stimulation of mitochondrial PKA in COX deficient cells with reduced COX activity, respiration, and ATP synthesis, resulted in a resetting and optimization of the balance between energy metabolism, ROS production, and OXPHOS biogenesis.

As a bicarbonate and Ca\(^{2+}\) sensor, intra-mitochondrial sAC may couple the activity of the TCA cycle (main source of CO\(_2\) in the cell) as well as alterations in the mitochondrial Ca\(^{2+}\) concentration with the OXPHOS activity and the rate of ATP synthesis. This is of particular importance for the appropriate matching of myocardial work with energy supply: increased cardiomyocytes activity, accompanied by the rise of cytosolic and mitochondrial Ca\(^{2+}\) concentration, leads to the activation of sAC/cAMP/PKA pathway resulting in a higher rate of ATP synthesis. Aside from the physiological role of sAC, one may suppose that it plays a pro-survival role in cardiac pathologic conditions by promoting the activation of oxidative respiration and ATP synthesis. Thus, strategies directed toward the enhancement of the first sAC-function in mitochondria may contribute to prevent several myocardial pathologies, including reperfusion-induced injury.
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Figure 1.3 sAC/cAMP/PKA pathway in mitochondria. Activation of sAC by Ca$^{2+}$ and HCO$_3^-$ triggers the phosphorylation of complex I and IV through PKA, leading to an increase in COX activity, membrane potential ($\Delta\psi$), O$_2$ consumption and ATP production without altering ROS level. Other OXPHOS complexes along with proteins related to TCA cycle, calcium homeostasis, apoptosis, transporters, fatty acid oxidation, and $\beta$-oxidation are also phosphorylated, but whether this involves sAC pathway is still unknown (Valsecchi F et al., 2014).
# Material and methods

## 2.1 Ethics statements

All animal experiments were performed in accordance with German animal welfare laws and were approved by the Animal Welfare Officer of the Justus Liebig University Giessen (Registration No: 530_M and 589_M). The animal housing facility was licensed by the local authorities (Az: FD62-§11JLUHumPhys). The methods used to euthanize the rats were consistent with the recommendations of the AVMA Guidelines for the Euthanasia of Animals.

## 2.2 Cellular models of investigation

To study the effect of mitochondrial sAC on ischemia/reperfusion injury (I/R), two *in vitro* models of simulated I/R were used.

1. Primary adult rat cardiomyocytes were subjected to simulated-I/R to investigate the effects of inhibition or activation of sAC using KH7 and 22 mmol/l bicarbonate, respectively.

2. Embryonic cardiomyoblasts H9C2 were subjected to a similar model of I/R to investigate effects of inhibition (KH7) and overexpression of sAC.

## 2.3 The Langendorff system's concept and the isolation of cardiomyocytes

Oscar Langendorff developed and described in the early 1900s the model of isolated retrograde perfused myocardium (Zimmer HG, 1998; Skrzypiec-Spring M et al., 2007) (Figure 2.1). The heart is perfused in a reverse mode via a cannula placed in the ascending aorta. The backwards pressure causes the aortic valve to close, forcing the perfusion buffer into the coronary vessels, carrying nutrients and oxygen to the cardiac muscle.
Material and methods

Figure 2.1 The Langendorff perfusion system. The apparatus used to isolate rat cardiomyocytes is shown with the magnification of a rat heart mounted on the cannula of the perfusion system.

200-300 g Wistar rats were purchased from Janvier Labs and anesthetized using 4.5% isoflurane before euthanasia by cervical dislocation. Chest was opened and the excised hearts were transferred into a large petri dish containing ice-cold salt solution (0.9% NaCl) and washed, and finally mounted on the cannula of the system. The perfusion medium was calcium free Powell’s buffer containing 110 mmol/l NaCl, 2.6 mmol/l KCl, 1.2 mmol/l KH₂SO₄, 1.2 mmol/l MgSO₄, 25 mmol/l HEPES, 11 mmol/l glucose, gassed with O₂. To allow digestion of the heart tissue, 5 mg collagenase (CLS-2, Worthington Biochemical Corporation) was added to the buffer with 25 µmol/l CaCl₂. Subsequently, digested hearts were chopped. The tissue was filtered through 200 µm nylon mesh gauze and centrifuged for 1 minute at 25 g. Then, cells underwent two more resuspension and centrifugation steps with increasing CaCl₂ concentration. Cells were finally resuspended with culture medium 199 (M-199, Biochrom) supplemented with 5% fetal calf serum (FCS), 5 mmol/l
creatin (Sigma), 2 mmol/l carnitine (Sigma), 5 mmol/l taurine (Sigma), 10 µmol/l cytosine-arabinofuranoside (Sigma), 2% penicillin/streptomycin (CTT medium), pH 7.4 at 37°C.

2.4 Culture of adult rat cardiomyocytes

Petri dishes were pre-incubated overnight with 0.75 µg/ml laminin (Roche) in CCT medium under CO₂-free atmosphere at 37°C. Cells were seeded and incubated. One hour after plating, cells were washed with CCT FCS-free medium. As a result of the wash, dead cells were removed, leaving a homogeneous population of rod-shaped cardiomyocytes attached on the bottom of the dish (Figure 2.2). Only cells exhibiting a rod-shaped morphology and no signs of sarcolemmal blebbing were used for imaging experiments.

Figure 2.2 Cardiomyocyte culture. Representative micrograph of cultured adult rat cardiomyocytes by phase contrast imaging.
2.5 Culture of embryonic cardiomyoblast cell line H9C2

Embryonic cardiomyoblast cell line H9C2 was originally derived from embryonic rat ventricular tissue (Kimes BW and Brandt BL, 1976). H9C2 cells have been extensively used in cardiologic research. Although H9C2 cells are no longer able to beat and are a proliferating cell line, they show many similarities to primary cardiomyocytes, including electrophysiological properties (Hescheler J et al. 1991; Sipido KR and Marban E, 1991), and the expression of titin (Van der Loop et al., 1996), myosin heavy chain, SERCA, calsequestrin-2 (Lenço J et al., 2015), and L-type Ca\(^{2+}\) channels (Sipido KR and Marban E, 1991). Rat cardiac myoblast H9C2 were obtained from ATCC (CRL-1446) and cultured in high glucose (4500 mg/l) Dulbecco’s modified Eagle’s medium (DMEM, Life technologies) supplemented with 10% FCS and 1 mmol/l sodium pyruvate (Biochrom) in a 5% CO\(_2\) incubator (Figure 2.3). Sub-confluent cells (60-70%) were sub-cultured 1:3 to prevent loss of myoblastic cells as recommended by the provider. Two days before experiments, cells were cultivated with 2.5% FCS.

![Cardiomyoblast cell culture](image)

**Figure 2.3 Cardiomyoblast cell culture.** Representative micrograph of cultured H9C2 cells by phase contrast imaging.
2.6 Loading of Fura-2 and BCECF

To measure cytosolic Ca\(^{2+}\) fluctuations, cardiomyocytes and H9C2 cells were loaded with 2.5 \(\mu\)mol/l acetoxymethyl ester (AM) Fura-2 (Thermo Fisher Scientific) in FCS-free culture medium for 45 minutes at 37°C. Cells were then post-incubated with fresh CCT medium for 20 minutes to allow the hydrolysis of the acetoxymethyl esters within the cell. To measure cytosolic H\(^+\) concentration, cardiomyocytes were loaded with 0.5 \(\mu\)mol/l AM-BCECF in CCT medium for 5 minutes at 35°C. Cells were then washed twice and post-incubated with 5% FCS CCT medium for 30 minutes at 35°C. The fluorescence of Fura-2- and BCECF-loaded cells was respectively ~10 and ~5 times higher than the background.

2.7 Media

The perfusion chamber (1 ml filling volume) placed on the microscope stage was perfused at a flow rate of 0.5 ml/min with Tyrode's solutions modified for each experimental setting:

- \(\text{HEPES (0\% CO}_2\): (mmol/l)}\) 125 NaCl, 2.6 KCl, 1.2 \(\text{KH}_2\text{PO}_4\), 1.2 MgSO\(_4\), 25 HEPES.
- \(22 \text{mmol/l bicarbonate (5\% CO}_2\): (mmol/l)}\) 96 NaCl, 2.6 KCl, 1.2 \(\text{KH}_2\text{PO}_4\), 1.2 MgSO\(_4\), 22 NaHCO\(_3\), 25 HEPES; equilibrated with 5% CO\(_2\), 21% O\(_2\), air.

Before the experiments, 1.2 mmol/l CaCl\(_2\) and 5 mmol/l glucose were added in the buffers; pH was 7.4 at 36°C. To mimic the ischemic condition, a metabolic inhibition (chemical anoxia) was applied using 2 mmol/l sodium cyanide (NaCN) in glucose-free medium, pH=6.4. Chemical anoxia (CA) in H9C2 cells was performed by the further addition of 5 mmol/l 2-deoxy-D-glucose (2-DOG). Chemical anoxia/reperfusion (CA/R) injury was obtained by superfusing cells with normoxic buffer after CA.

2.8 \(\text{Ca}^{2+}\) and pH measurements

Coverslips with loaded cardiomyocytes and H9C2 cells were introduced into a gastight, temperature-controlled (36 and 35°C, respectively), transparent perfusion chamber positioned on the stage of a microscope (Olympus IX-70, Hamburg, Germany) adapted to a Video-Imaging-System (Visitron Systems, Puchheim, Germany), containing a light source (Polychrome V) on the excitation side and a CCD Camera (Retiga 2000-RV, QImaging, Surrey, Canada) on the fluorescence detection side. Data were analyzed using VisiView Software (Visitron Systems, Germany) (Figure 2.4). A field
with ~10 cells was chosen to perform the experiments. Cells were excited at wavelengths 340 and 380 nm for Fura-2 or 490 and 440 for BCECF detection and the emitted light from a 10 x 10 µm within a single cell was collected by the photomultiplier. Simultaneously to each fluorescence acquisition, images of cells were recorded by the camera and stored.

**Figure 2.4 Live imaging system.** The system includes a perfusion chamber on the stage of a microscope connected to polychrome V on the excitation side and to a camera which transfers the recordings to VisiView software. A temperature controller is connected to the microscope stage.

### 2.9 Fura-2 compartmentation

The loading protocols used were selected from a number of variations because they provided the highest yield in fluorescence and minimal dye compartmentation. To assess the extent of intracellular dye compartmentation, cells were chemically “skinned” with digitonin. First, cells were perfused for about 5 minutes with EGTA buffer, 135 mmol/l KCl, 5 mmol/l NaCl, 5 mmol/l HEPES, 1 mmol/l EGTA, then they were metabolically inhibited by the addition of 1 mmol/l KCN to prevent hypercontraction and superfused for about 10 minutes. After this procedure 3 µmol/l digitonin was
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added to KCN buffer. Digitonin permeabilizes the sarcolemmal membrane leaving organelles intact (Altschuld RA et al., 1985b). After the release of the dye from the cytosol, the residual fluorescence was measured, which was a sum of fluorescence from compartments and background fluorescence. To separate them, 1 mmol/l MnCl$_2$ and 5 µmol/l ionomycin were added to KCN buffer. This quenched the fluorescence of the dye within the organelles leaving background fluorescence (Borzak S et al., 1990) (Figure 2.5). The background’s fluorescence was subtracted from the initial fluorescence. Excitation of Fura-2 was done at 360 nm every 3000 milliseconds. We found that loading of 2.5 µmol/l Fura-2 at 36°C for cardiomyocytes and 35°C for H9C2 cells were the optimal conditions to obtain a stable Ca$^{2+}$ signal and low organelles compartmentation (~10%).

![Representative Fura-2 compartmentation experiment in H9C2 cells.](image)

(A) Images show Ca$^{2+}$ independent Fura-2 fluorescent signal before and after the addition of digitonin, and after the addition of MnCl$_2$. (B) Time course of Fura-2 fluorescence at 360 nm in H9C2 cells undergoing compartmentation experiment.
2.10 BCECF calibration

BCECF calibration was done by exposing cardiomyocytes to 1 µmol/l nigericin and 10 µmol/l valinomycin, which are K⁺-H⁺ and K⁺ ionophores, respectively. Cells were perfused with a modified Tyrode's solution containing 10 mmol/l NaCl, 125 mmol/l KCl, 1.2 mmol/l MgSO₄, 25 mmol/l HEPES, 0.5 mmol/l EGTA, 5 mmol/l Glucose, 1 µmol/l nigericin, 10 µmol/l valinomycin, at different pH values: the sequence was pH 6, 6.5, 7, 7.5 at 36°C (Figure 2.6 A). Excitation was done at 490 and 440 nm and images were acquired every minute. Means of BCECF ratio were used to generate a calibration curve used for the normalization of pH data through the equation y=1.7898ln(x)+3.9341 (Figure 2.6 B).

![Figure 2.6 BCECF calibration. (A) Representative original recording of BCECF ratio in cardiomyocytes under different intracellular pH. (B) BCECF calibration curve used for the conversion of BCECF ratio to pH value.](image)

2.11 Analysis of amplitude and frequency of Ca²⁺ oscillations, diastolic level of cytosolic Ca²⁺, and cell hypercontraction

In Ca²⁺ live imaging experiments with cardiomyocytes, images were acquired every 3000 milliseconds during the anoxic period and every 300 milliseconds during the reperfusion period. At the end of each experiment, data were used to analyze amplitude and frequency of Ca²⁺ oscillations, and diastolic level of cytosolic Ca²⁺. Changes in cardiomyocytes length were determined from recordings of each live imaging experiment. Hypercontraction was calculated as the result of the difference between the length after rigor occurred and the length at 10 minutes of
reperfusion divided by the full length of the cell. In the case of hypercontracted cells, the dimension of the cell was determined along its previous axis.

H9C2 cells did not show any oscillatory event, therefore, only the diastolic level of cytosolic Ca\textsuperscript{2+} was analyzed.

2.12 Quantification of cell death

To quantify cellular death, cells were stained with DAPI and 1 µg/ml Propidium Iodide (PI). At the end of each experiment, cells were incubated for 1 hour in normoxic buffer. Then, cardiomyocytes and H9C2 were stained with DAPI and PI at 37°C. For quantitative assay, a blind analysis of 200-300 cells from nine randomized fields was used. Cells were scored as dead when at least one nucleus produced unequivocal bright red fluorescence due to membrane disruption that allowed the dye entering the cell (Figure 2.7).

![Necrotic staining](image)

**Figure 2.7 Necrotic staining.** Representative image of PI and DAPI staining on cardiomyocytes.
2.13 Electroporation

H9C2 cells were seeded onto 24 mm diameter glass coverslips. Transfection of FRET EPAC-based H30 cAMP-sensors was performed at 40–50% confluence by electroporation with Amaxa™ Nucleofector™ II applying T-20 program according to manufacturer instructions. 200,000 cells were mixed with 3 µg DNA and transferred into electroporation cuvette with transfection buffer 5 mmol/l KCl, 15 mmol/l MgCl₂, 50 mmol/l mannitol, 120 mmol/l Na₃HPO₄/NaH₂PO₄, pH=7.2. After electroporation cells were cultured in DMEM 10% FCS for 24 hours, followed by 24 hours starvation with DMEM 2.5% FCS. Live imaging experiments were performed 48 hours after transfection.

2.14 Fluorescence resonance energy transfer imaging (FRET)

H9C2 cells were transfected with nucleus- and mitochondria-targeted EPAC-based cAMP-sensor, kindly provided by Dr. Giulietta Di Benedetto (Institute of Neuroscience, CNR, Padova, Italy). The mitochondria-targeted version of EPAC-based cAMP sensor was obtained by fusing repetitive targeting sequences from subunit VIII of the human COX at the N terminus (Di Benedetto G et al., 2013). The nuclear-targeted version of EPAC-based cAMP sensor was generated by fusion of the nuclear localization signal PKKKRKVEDA (nls) at the C terminus (Di Pilato M et al., 2004). Binding of cAMP to the cAMP binding domain results in conformational changes which cause an increase in the distance between cyan fluorescence protein (CFP) and yellow fluorescence protein (YFP), followed by changes in FRET signal. Cells were maintained at room temperature (RT) (20°C-22°C) in HEPES-buffered Ringer-modified saline 5 mmol/l KCl, 1 mmol/l Na₃PO₄, 1 mmol/l MgSO₄, 20 mmol/l HEPES, 110 mmol/l NaCl supplemented with 2 mmol/l CaCl₂ and 5 mmol/l glucose, Images were acquired every 6 seconds with 40x objective and processed with VisiView software. FRET changes were measured as changes in the background-subtracted 470/530 nm fluorescence emission intensities upon excitation at 430 nm.

2.15 Analysis of lactate dehydrogenase in culture medium

Lactate dehydrogenase (LDH) activity in cell culture medium was used as an indicator for necrosis and was determined using Cytotoxicity Detection Kit (Roche Applied Science). LDH is a cytosolic enzyme that is released into the media from damaged cells. Culture medium was centrifuged at
800 g for 5 minutes at 4°C, loaded into a 96 wells plate, and then incubated with the reaction mixture for 30 minutes at RT. After incubation the absorbance of samples was measured at 492 nm with ELISA reader (TECAN Infinite M200).

### 2.16 Cellular cAMP analysis

Analysis of the total cellular cAMP content was performed applying the direct cAMP Enzyme Immunoassay Kit (Enzo Life Sciences). After treatment, cells were lysed and the lysate was loaded into 96 wells coated with GxR IgG antibody. A polyclonal antibody to cAMP bound in a competitive manner the cAMP in the sample or conjugate. When the substrate was catalyzed by the alkaline phosphatase on the cAMP conjugate a yellow color was generated. Therefore, the signal was inversely proportional to the amount of cAMP in the sample. The absorbance was measured at 405 nm with ELISA reader (FLUOstar OPTIMA, BMG Labtech).

### 2.17 sAC overexpression in H9C2 cells

Plasmids for the untargeted expression of sAC, predominantly in the cytosol (cyto-sAC), mitochondria-targeted sAC (mito-sAC), and GFP were kindly provided by Dr. Yury Ladilov (Center for Cardiovascular Research, Charité, Berlin, Germany). Cyto-sAC, mito-sAC, and GFP clones were generated by lipofection using the jetPRIME transfection reagent (Polyplus). H9C2 cells were seeded into 6 well plates (~70,000 cells per dish) and two days after were transfected with 2 µg DNA in M199 10% FCS. Cells were incubated at 37°C for 24 hours and then washed with M199 3% FCS and incubated for 2 days. sAC expression in H9C2 cells transfected with GFP, cyto-sAC, or mito-sAC plasmids was analyzed by western blotting.

### 2.18 Protein measurements and western blotting

Western blotting was applied to verify the occurred overexpression of cyto-sAC and mito-sAC plasmids in H9C2 cells, using GFP-expressing cells as control. Cells were lysed in lysis buffer containing 1% SDS, 10 mmol/l EDTA, 50 mmol/l Tris, 20 mmol/l NaF, 0.5 mmol/l PMSF, 1 µmol/l leupeptin, 0.15 µmol/l aprotinin. Equal amounts of total proteins (20-30 µg/well) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The primary antibody used was rabbit anti-sAC (clone K2, kindly provided by Dr. S. Kumar, Justus-Liebig-Universität, Gießen,
Germany). Specific band at 50 kDa was visualized by chemiluminescence detection applying horseradish peroxidase (HRP)–conjugated secondary antibody, Clarity Western ECL Blotting kit (Bio Rad). Equal sample loading was confirmed by a second treatment with mouse antibodies against vinculin (Sigma).

2.19 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 (ANOVA) followed by the Holm–Sidak post hoc test. Statistical significance was accepted when P<0.05.
3 Results

3.1 Characterization of reperfusion-induced injury in adult rat cardiomyocytes

The reperfusion of the heart after prolonged period of ischemia can aggravate the ischemic injury of the myocardium. Calcium plays a pivotal role in this dramatic event. Indeed, at the time of reperfusion when the cell is re-energized, SERCA starts to uptake Ca$^{2+}$ which accumulates inside the sarcoplasmic reticulum. The excess of Ca$^{2+}$ is released into the cytosol, generating transient high cytosolic Ca$^{2+}$ concentrations that have two principle effects: (i) they lead to the uncontrolled hypercontracture, and (ii) they promote accumulation of Ca$^{2+}$ in mitochondria leading to the opening of mitochondrial permeability transition pores, a main cause of mitochondrial injury and dysfunction (e.g. Ladilov YV et al., 1997; Abdallah Y et al., 2005; Abdallah Y et al., 2006, Abdallah 2011).

It is known that the inhibition of mitochondrial cytochrome oxidase (COX) is one of the earliest events occurring under anaerobic conditions. Inorganic cyanides, such as sodium cyanide may simulate this effect of anoxia. Their action is related to the presence of a negatively charged cyanide ion which can inhibit COX (Adler M et al., 1999; Nelson L, 2006). Cyanide anion binds to the iron within this enzyme preventing the transport of electrons from COX to oxygen. As a result the electron transport chain is blocked and the cell is no longer able to produce ATP through the aerobic metabolism. This phenomenon is called histotoxic hypoxia, which refers to the inability of cells to use oxygen to generate energy, despite the physiological delivery of oxygen to cells or tissue. Thus, inorganic cyanides mimic the metabolic inhibition induced by ischemia. Indeed, sodium cyanide (NaCN) has been widely used as a model for simulated-ischemia in the brain, heart, and eye (Bernaudin M et al., 1998; Kitagawa H et al., 2008; Kokona D et al., 2012). Furthermore, the arrest of cellular respiration by NaCN is highly reproducible. To optimize the chemical anoxia (CA) protocol with NaCN in this study, several experimental parameters were varied, e.g., temperature, time of Fura-2 loading, and time of CA. The optimal CA time was found to be 30-35 minutes, since it allowed the recovery from Ca$^{2+}$ overload, i.e., metabolic competence, of about 50% cells. Finally, the 15 minutes reperfusion time allowed the complete recovery of Ca$^{2+}$ homeostasis.
To characterize the ability of cells to recover from CA, the following parameters were used:

- Amplitude and frequency of spontaneous Ca^{2+} oscillations;
- Diastolic level of cytosolic Ca^{2+};
- Number of cells, which did not recover or lost Ca^{2+} control during 10 minutes of reperfusion (viability);
- Number of necrotic cells defined by positive propidium iodide staining (necrosis).

Accordingly, Ca^{2+} oscillations were analyzed only in alive cells. Two examples of Ca^{2+} signal during reperfusion in cardiomyocytes undergoing CA/R are given in Figure 3.1. In one case the cell completely recovered during 15 minutes of reperfusion, i.e., Ca^{2+} reaches control, pre-anoxic level. In contrast, the other cell lost Ca^{2+} control after 10 minutes reperfusion, indicating cell death.

**Figure 3.1 Representative Ca^{2+} signal in cardiomyocytes undergoing CA/R.** (A) Morphology of the cells is presented at the time of normoxia, end of anoxia, and after 10 minutes of reperfusion. Myocytes length is reduced during CA due to rigor contracture. With the onset of reperfusion cardiomyocytes develop hypercontracture. Original recordings of cytosolic Ca^{2+} (Fura-2 ratio) during CA and reperfusion in (B) cell which recovered Ca^{2+} homeostasis during reperfusion and (C) cell which lost metabolic control leading to sustained Ca^{2+} overload after 10 minutes of reperfusion.
3.2 Role of sAC in reperfusion-induced injury of cardiomyocytes

sAC was recently found to localize in mitochondria and to control phosphorylation/activity of different proteins in the respiratory chain, OXPHOS activity, and ATP synthesis (Achin-Perez R et al., 2009a-b; Di Benedetto G et al., 2013, De Rasmo D et al., 2015). This might be important during initial phase of reperfusion, i.e., during recovery of mitochondrial function. Unfortunately chemical compounds which might act as sAC agonists are currently not available, thus we adopted the strategy to use bicarbonate, a natural activator of sAC (Cann MJ et al., 2003; Steegborn C et al., 2005). In this study the physiological concentration of 22 mmol/l bicarbonate was chosen because it has no significant effect on pH variations in our model. Moreover, it was shown that in concentration 22 mmol/l bicarbonate leads to about 70% of the maximal sAC activation, (Geng W et al., 2005).

To investigate the effect of sAC activation operated by bicarbonate in reperfusion-induced injury, the effects of CA/R performed in bicarbonate-free, HEPES-buffered medium (HEP/HEP), were compared with effects of CA/R performed in bicarbonate-buffered (22 mmol/l) medium (BIC/BIC).

Applying bicarbonate during CA/R significantly reduced amplitude and frequency of the spontaneous Ca\(^{2+}\) oscillations at the onset of reperfusion, which was accompanied by significantly accelerated recovery of diastolic Ca\(^{2+}\) concentration (Figure 3.2 A-C). Accordingly, the viability of cardiomyocytes during reperfusion was significantly improved. No significant effect of bicarbonate during CA/R on cardiomyocytes hypercontracture and necrosis has been found (Figure 3.3 A-C).

Acidosis during reperfusion has been shown to ameliorate the reperfusion-induced injury (Ladilov YV et al., 1995). To prove the role of pH in the protective effects of bicarbonate, cytosolic pH has been analyzed applying fluorescent dye BCECF. The absolute pH values were obtained through calibration curve and applied to experimental data. Despite the initial difference in pH between the two experimental groups at the time of normoxia, pH levels both during CA and reperfusion were not significantly different between the groups (Figure 3.2 D). Thus, the observed protective effect of bicarbonate is not due to cytosolic pH alterations at the time of CA/R. The difference between the groups at the time of normoxia is likely an artifact due to the Fura-2 loading protocol. Indeed, the change from the loading HEPES-buffered medium to the bicarbonate-buffered medium may shortly cause a decrease in pH.
Results

Figure 3.2 Presence of bicarbonate in superfusion medium during CA and reperfusion improves recovery of Ca\(^{2+}\) homeostasis. Time courses of (A) amplitude of Ca\(^{2+}\) oscillations, (B) frequency of Ca\(^{2+}\) oscillations, and (C) diastolic Ca\(^{2+}\) level in cardiomyocytes undergoing 30 minutes CA and 15 minutes reperfusion in HEPES (HEP/HEP) or 22 mmol/l bicarbonate (BIC/BIC) buffered medium. (D) Time course of cytosolic pH in cardiomyocytes during CA/R. N indicates normoxia. Data are means ± SEM, (A, B, C) n=4, (D) n=3. *, P<0.05, BIC/BIC vs. HEP/HEP.

Figure 3.3 Presence of bicarbonate in superfusion medium improved viability, but has no significant effect on reperfusion-induced hypercontracture and necrosis. (A) Viability and (B) reperfusion-induced hypercontracture were measured at 10 minutes of reperfusion, whereas (C) reperfusion-induced necrosis was analyzed after 1 hour of reperfusion. Data are means ± SEM, n=4. *, P<0.05, BIC/BIC vs. HEP/HEP.
3.3 Effect of sAC activation during reoxygenation on the reperfusion-induced injury

Activation of sAC during reoxygenation with 22 mmol/l bicarbonate

Although presence of bicarbonate during CA/R supports activity of sAC, the depletion of ATP during CA most likely disturbs cAMP synthesis (Zippin JH et al., 2013), whereas recovery of ATP during reperfusion will recover cAMP synthesis. Thus, to check whether the protection against reperfusion-induced injury may also be observed by stimulation of cyclase with bicarbonate only during reperfusion, the additional protocol has been applied. In this protocol, reperfusion after CA in HEPES-buffered medium has been performed at the absence (HEP/HEP) or presence of 22 mmol/l bicarbonate (HEP/BIC) equilibrated with 5% CO₂. Under this protocol sAC should be activated at the beginning of reperfusion. Imaging of nuclear and mitochondrial cAMP applying FRET-based cAMP sensor in H9C2 cells demonstrated a rapid rise of cAMP under application of bicarbonate (Figure 3.4 B).

Figure 3.4 cAMP content in H9C2 cells exposed to 50 mmol/l bicarbonate. (A) Images of nucleus and mitochondria of H9C2 cells expressing the EPAC-based cAMP sensor. (B) Representative original recordings of cAMP generation in nucleus and mitochondria of H9C2 cells subjected to the increase of bicarbonate concentration from 0 to 50 mmol/l.
Analysis of cytosolic Ca\(^{2+}\) during reperfusion phase did not reveal significant difference in amplitude/frequency of Ca\(^{2+}\) oscillations or in diastolic calcium between groups (Figure 3.5 A-C). Similarly, reperfusion of cardiomyocytes in bicarbonate buffer did not affect viability and hypercontracture. In contrast, the presence of bicarbonate during reperfusion significantly attenuated necrotic cell death (Figure 3.6 A-C). Again, to ensure that the beneficial effect of bicarbonate against necrosis was not due to acidosis, cytosolic pH was measured in cells subjected to HEP/HEP or HEP/BIC protocols. Despite the difference in pH between the two experimental groups at 30 minutes time point (CA), pH levels both during CA and reperfusion were not significantly different between the groups. The difference at 30 minutes time point was unexpected, since conditions during CA were the same in the two groups (Figure 3.5 D). Therefore, this discrepancy may not be taken into account.

![Figure 3.5 Presence of bicarbonate in superfusion medium during reperfusion does not affect recovery of Ca\(^{2+}\) homeostasis.](image)

**Figure 3.5** Presence of bicarbonate in superfusion medium during reperfusion does not affect recovery of Ca\(^{2+}\) homeostasis. Time course of (A) amplitude of Ca\(^{2+}\) oscillations, (B) frequency of Ca\(^{2+}\) oscillations, and (C) diastolic Ca\(^{2+}\) level in cardiomyocytes undergoing 30 minutes CA and 15 minutes reperfusion in HEP/HEP and HEP/BIC protocols. (D) Time course of cytosolic pH in cardiomyocytes during CA/R. N indicates normoxia. Data are means ± SEM, (A, B, C) n=6, (D) n=3. *, P<0.05, HEP/BIC vs. HEP/HEP.
Results

Figure 3.6 Presence of bicarbonate in reperfusion medium reduces reperfusion-induced necrosis, but has no effect on viability and hypercontracture. (A) Viability and (B) reperfusion-induced hypercontraction were measured at 10 minutes of reperfusion, whereas (C) reperfusion-induced necrosis was analyzed after 1 hour of reperfusion. Data are means ± SEM, n=7. *, P<0.05, HEP/BIC vs. HEP/HEP.

3.4 Effect of sAC inhibition during reoxygenation on reperfusion-induced injury in cardiomyocytes

Effect of KH7 on cardiomyocytes

To substantiate the role of sAC in reperfusion-induced injury, sAC activity was suppressed by treatment with the selective sAC-inhibitor KH7. 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 tmAC and soluble guanylyl cyclase up to 100 μmol/l (Hess KC et al., 2005). In normoxic cardiomyocytes, KH7 dose-dependently reduced cellular cAMP content with maximal effect of about 50% at 30 μmol/l (Figure 3.7 A). When applied for 30 minutes in normoxic cardiomyocytes at 30 μmol/l, KH7 had no cytotoxic effect both in HEPES and bicarbonate buffers, although KH7 started to become cytotoxic after one hour treatment (Figure 3.7 B). Since KH7 has been applied during reoxygenation in our experiments only during 15 minutes, the potential cytotoxic effect at this time window can be excluded.
Inhibition of sAC during reperfusion

Applying CA/R protocol in bicarbonate-buffered medium, we found that suppression of sAC activity during reperfusion with 30 µmol/l KH7 significantly affected the ability of cardiomyocytes to recover the cytosolic Ca\textsuperscript{2+} control. Representative original recordings of cytosolic Ca\textsuperscript{2+} show an example of short recovery of cytosolic Ca\textsuperscript{2+} control followed by cell death in KH7 treated group (Figure 3.8 B). Statistical analysis show significant disturbance of Ca\textsuperscript{2+} homeostasis recovery indicated by elevated diastolic Ca\textsuperscript{2+} and nearly prevented Ca\textsuperscript{2+} oscillations, i.e., ability of SERCA to uptake cytosolic Ca\textsuperscript{2+} (Figure 3.9 A-C). Such failure to maintain the Ca\textsuperscript{2+} homeostasis is likely due to disturbed recovery of energy control followed by cell death. Indeed, KH7 treatment significantly reduced viability and increased necrosis of reperfused cardiomyocytes (Figure 3.10 A, B). Therefore, the basal activity of sAC is crucial for recovery of Ca\textsuperscript{2+} homeostasis and, therefore, for survival of cardiomyocytes during reperfusion.
Figure 3.8 Representative recordings of cytosolic Ca\(^{2+}\) (Fura-2 ratio) in single cardiomyocytes during reperfusion in bicarbonate-buffered medium. (A) Example of two cardiomyocytes undergoing normoxic superfusion in presence (red) or absence (blue) of KH7. (B) Example of a cardiomyocyte undergoing reperfusion with 30 µmol/l KH7 after chemical anoxia. Note, initial ability of cell to recover Ca\(^{2+}\) homeostasis was lost within 5 minutes reperfusion indicating cell death.

Figure 3.9 Inhibition of sAC during reperfusion prevented recovery of Ca\(^{2+}\) homeostasis. Time course of (A) amplitude of Ca\(^{2+}\) oscillations, (B) frequency of Ca\(^{2+}\) oscillations, and (C) diastolic Ca\(^{2+}\) level in cardiomyocytes undergoing 30 minutes CA and 15 minutes reperfusion in bicarbonate buffered medium (22 mmol/l) at the absence (control) or presence (KH7) of 30 µmol/l KH7. Control indicates treatment with solvent. Data are means ± SEM, n=7, * \(P<0.05\), KH7 vs. Control.
Establishment of reperfusion-induced injury in embryonic cardiomyoblast cell line H9C2

Previous reports demonstrated the localization of sAC in mitochondria (Acin-Perez R et al., 2009a-b) and suggested the importance of intra-mitochondrial sAC-PKA axis in the regulation of OXPHOS activity (Acin-Perez R et al., 2009a-b; Di Benedetto G et al., 2013). Mitochondrial function is essential for rapid recovery of energy and ion homeostasis in reperfused cardiomyocytes. Therefore, to further strengthen the finding demonstrating the beneficial role of sAC in protection against reperfusion-induced injury, we aim to prove whether an increase of sAC expression in mitochondria may protect against reperfusion-induced injury.

For this purpose rat embryonic cardiomyoblast cell line (H9C2) was used. To validate the model, first, effect of sAC suppression with KH7 was examined in these cells treated with 20 minutes CA followed by reperfusion. Similar treatments with normoxic medium have been performed in control cells.

In normoxic H9C2 cells, treatment with KH7 for 3 hours dependently reduced cellular cAMP content with maximal effect of about 40% at 30 µmol/l (Figure 3.11 A), whereas KH7 had no cytotoxic effect within 3 hours (Figure 3.11 B).
In H9C2 cells no spontaneous oscillations during reperfusion could be observed. Thus, only mean Ca^{2+} concentration was examined. Similarly to cardiomyocytes after CA, the suppression of sAC activity, with 30 µmol/l KH7 during reperfusion in H9C2 cells disturbed the recovery of Ca^{2+} homeostasis, indicated by elevation of Fura-2 ratio during reoxygenation (Figure 3.12 A). In accordance, KH7 treatment significantly increased necrosis of reperfused H9C2 cells (Figure 3.12 B), suggesting that the inability to control cytosolic Ca^{2+} in presence of KH7 is due to disturbed recovery of energy control. Thus, the activity of sAC seems to be important for recovery of Ca^{2+} homeostasis and for survival of cardiomyoblasts during reperfusion.
Results

3.6 Establishment of untargeted and mitochondria-targeted sAC overexpression in H9C2 cells

The similarities of data obtained in cardiomyocytes and H9C2 cells under sAC inhibition encouraged us to investigate the effect of sAC overexpression in cardiomyoblasts, discriminating between mitochondria-targeted (mito-sAC) and non-targeted, cytosolic (cyto-sAC) overexpression of sAC. FACS analysis of the transfection efficacy using GFP-expressing cells revealed a maximal transfection efficacy of about 45% after 24 hours transfection with 2 µg DNA versus the 40% efficacy obtained after 48 hours transfection (data not shown). Representative images of transfection efficacy in H9C2 cells are given in Figure 3.13. The quantification of sAC plasmids expression showed a more than 2-fold increase in cyto-sAC and mito-sAC (Figure 3.14). The specificity of mitochondrial sAC targeting was confirmed by cell fractionation and western blot analysis of sAC expression in cytosolic and mitochondrial fractions (data not shown).

Figure 3.12 Presence of 30 µmol/l KH7 in reperfusion medium aggravates reperfusion-induced injury. (A) Time course Ca$^{2+}$ level in cardiomyoblasts exposed to reperfusion at the presence (KH7) or absence (control) of KH7. (B) reperfusion-induced necrosis in cardiomyoblasts after exposure to CA or normoxia for 20 minutes followed by reperfusion at the presence (KH7) or absence (control) of KH7 are here presented. Data are means ± SEM, (A) n=7, (B) normoxia n=3, CA/R n=10. (A) *, P<0.05, vs. Control. (B) *, P<0.05, vs. KH7 in Normoxia; #, P<0.05, vs. Control in CA/R.
Recent reports demonstrated the positive effect of mito-sAC expression on mitochondrial function in various cell types (Acin-Perez R et al., 2009a-b; Di Benedetto G et al., 2013). To study the effect of sAC overexpression on reperfusion-induced cell death, different CA incubation time followed by 1 hour reperfusion were analyzed. The optimal protocol consisting of 2 hours CA followed by 1 hour reperfusion was found to clearly demonstrate the reperfusion-induced injury, i.e., an increase of LDH activity only during 1 hour reperfusion (Figure 3.15 A). The overexpression of
mito-sAC markedly reduced reperfusion-induced rise in LDH release and number of necrotic cells. In contrast, overexpression of cytosolic sAC only moderately reduced cell death (Figure 3.15 B, C). Taken together, these data suggest that mitochondrial sAC activity is important to protect cells against cell death after CA/R.

Figure 3.15 Overexpression of mitochondria-targeted sAC protects against reperfusion-induced cell death. (A) The effect of 2 hours and 4 hours of CA and 1 hour reperfusion is compared to the effect of equal treatment under normoxia. Blue bars represent the total amount of LDH released during normoxia 4 hours and CA 2-4 hours, red bars represent the total amount of LDH released only during 1 hour of reperfusion. (B,C) The effects of GFP, cyto-sAC or mito-sAC expression on (B) LDH activity and (C) necrosis in control, normoxic (Normoxia) H9C2 cells or cells treated with CA/R are presented. Data are means ± SEM, (A-C) n=4. (A) *, P<0.05, vs. Normoxia w/o reperfusion. #, P<0.05, vs. Normoxia in reperfusion. $, P<0.05, vs. CA 2h/R in reperfusion. (B, C) *, P<0.05, vs. GFP in Normoxia. #, P<0.05, vs. GFP in CA/R. $, P<0.05, vs. cyto-sAC.
4 Discussion

The overall aim of this thesis was to investigate the role of sAC in reperfusion-induced injury applying two *in vitro* models of simulated ischemia and reperfusion in isolated adult rat cardiomyocytes and embryonic rat cardiomyoblasts.

The main findings are as followed:

1. The basal activity of sAC is necessary and sufficient to support the recovery of Ca\(^{2+}\) homeostasis and survival of cardiomyocytes during CA/R.

2. Intra-mitochondrial sAC plays a key role in the protection of H9C2 cells under CA/R, preventing reperfusion-induced necrotic cell death.

Despite our data still have to be confirmed by *in vivo* experiments, the model of isolated cardiomyocytes applied in this study allows the analysis of the reperfusion-induced injury independently of the effects of other cell types, mechanical forces and hormones. Embryonic cardiomyoblast cell line was used in this project to study the effect of sAC overexpression. Although H9C2 cells are no longer able to beat and are a proliferating cell line, they show many similarities to primary cardiomyocytes, including the expression of titin (Van der Loop et al., 1996), myosin heavy chain, SERCA, calsequestrin-2 (Lenčo J et al., 2015), L-type Ca\(^{2+}\) channels, and electrophysiological properties (Hescheler J et al. 1991; Sipido KR and Marban E, 1991). Furthermore, under simulated ischemia/reperfusion applied in this study, H9C2 cells developed reperfusion-induced cell death, which make them a suitable model to investigate the role of sAC in the reperfusion-induced injury.

The model of CA/R used in this study is based on a previous model developed for the investigation of ion homeostasis and hypercontracture (Ladilov YV et al., 1998). Conversely to the original model of simulated ischemia/reperfusion, in this study a metabolic inhibition with NaCN was applied to mimic anaerobic environment. NaCN treatment has been widely used as a model for simulated ischemia in the brain, heart, and eye (Bernaudin M et al., 1998; Kitagawa H et al., 2008; Kokona D et al., 2012).

The reperfusion of the heart after prolonged period of ischemia can aggravate the ischemic injury of the myocardium through several still incompletely understood mechanisms. The generation of
cytosolic Ca\textsuperscript{2+} oscillations, which in turn triggers the so called hypercontracture and the opening of MPTP, play here an essential role (Ladilov Y et al., 1997; Abdallah Y et al., 2005; Abdallah Y et al., 2006, Abdallah 2011). Thus, the ability of cells to recover Ca\textsuperscript{2+} homeostasis is extremely important for cell survival during reoxygenation and it is strongly based on the ability of mitochondria to produce sufficiently energy at the time of reperfusion (e.g. García-Dorado D et al., 1989; Ruiz-Meana M et al., 1999; Sanada S and Kitakaze M, 2004). Thus, interventions supporting mitochondrial function will beneficially affect cell survival after ischemic insult.

In the current study we hypothesized that sAC may significantly contribute to the recovery of mitochondrial function and, therefore, protection against reperfusion-induced injury. It has been shown that the mitochondria-localized sAC pool supports the activity of OXPHOS system due to PKA-dependent phosphorylation of different enzymes of the respiratory chain and it is evolutionary conserved, i.e., observed from yeast till mammals (Acin-Perez R et al., 2009a-b; Di Benedetto G et al., 2013; Hebert-Chatelain E et al., 2016; Hess KC et al., 2014). Furthermore, the transport of bicarbonate into mitochondria during ischemia was found to suppress mitochondrial apoptosis (Kumar S et al., 2011). As a bicarbonate and Ca\textsuperscript{2+} sensor, the intra-mitochondrial sAC may couple the activity of the tricarboxylic acid cycle as well as alterations in the mitochondrial Ca\textsuperscript{2+} concentration with the OXPHOS activity and the rate of ATP synthesis. This might be of particular importance in a condition of stress, like ischemia/reperfusion, where cells are ATP depleted.

To examine the role of sAC, two approaches have been applied, i.e., inhibition of sAC with specific inhibitor KH7, and activation of sAC either with bicarbonate or by overexpression. Treatment with KH7 at the time of reperfusion disturbed the recovery of Ca\textsuperscript{2+} homeostasis, triggering cell death, in both cardiomyocytes and H9C2 cells. This finding supported the hypothesis that sAC is specifically required at the time of reperfusion in order to protect cells against reperfusion-induced injury. In line with our findings, it has been shown that inhibition of intra-mitochondrial sAC by KH7 in hepatocytes supresses COX activity by 30\% and decreases ATP synthesis by 80\% (Acin-Perez R et al., 2009b). The authors also found that promotion of mitochondrial sAC activity by overexpression supports mitochondrial function. Therefore, we also examined whether promotion of sAC activity may support cell survival under CA/R. To address this issue, stimulation of sAC with bicarbonate in cardiomyocytes or sAC overexpression in H9C2 cells were used. The activation of sAC by
bicarbonate ameliorated the ability of cells to recover Ca\(^{2+}\) control during reperfusion in cardiomyocytes, showing a tendency in decreasing necrosis. In H9C2 cells, the overexpression of mitochondrial-targeted and untargeted sAC led to the protection against CA/R-induced cell death. Particularly, the protective effect was more evident when the intra-mitochondrial sAC was overexpressed, which nearly completely prevented necrotic cell death. Our data show, therefore, that sAC plays an important role in cell survival upon reperfusion-induced injury.

The presence of bicarbonate during CA and reperfusion seems to be important for recovery of Ca\(^{2+}\) homeostasis during reperfusion. Indeed, stimulation of sAC only during reperfusion, although reduced necrotic cell death, failed to improve recovery of Ca\(^{2+}\) homeostasis. Previous study in endothelial cells demonstrated that activity of mitochondrial sodium-bicarbonate symporter is important for protection against ischemia-induced mitochondrial injury (Kumar S et al., 2011). Thus, the sAC-independent protective mechanisms may be involved by presence of bicarbonate during CA.

The fact that inhibition of sAC only during reperfusion was sufficient to prevent recovery of Ca\(^{2+}\) homeostasis indicates that sufficient sAC activity is present in our model during reperfusion, even under bicarbonate-free conditions. Indeed, additionally to bicarbonate, Ca\(^{2+}\) is an important sAC activator. Since at the beginning of reperfusion high Ca\(^{2+}\) concentration is present in cardiomyocytes, one may suppose that sAC is sufficiently active and the additional stimulation with bicarbonate provides only moderate effect. In contrast, an increase of sAC expression may further contribute to the sAC-dependent protection as has been found in H9C2 cells expressing mitochondrial sAC.

In contrast to the acute necrotic cell death investigated here, previous reports suggest the importance of cytosolic rather than mitochondrial sAC in triggering apoptotic cell death in endothelial cells, cardiomyocytes and smooth muscle cells (Kumar S et al., 2009, Appukuttan A et al., 2012, Appukuttantan A et al., 2013) under various stress conditions, including simulated ischemia and reperfusion. In these studies translocation of sAC to mitochondria followed by mitochondrial Bax binding was described as a key signaling leading to activation of the mitochondrial pathway of apoptosis. In the current report a principally new role of sAC in cell death has been described, i.e.,
intra-mitochondria-localized sAC support survival of cell at the beginning of reperfusion. Thus, dependent on subcellular compartments, cytosol or mitochondria, sAC may play protective or detrimental role.

In conclusion, the present study supports the key role of sAC in reperfusion-induced injury. Particularly, the basal activity of sAC is necessary and sufficient to promote cardiomyocytes recovery of Ca^{2+} homeostasis and survival during reoxygenation. Furthermore, the promotion of sAC activity in mitochondria, rather than in cytosol, is important for protection against CA/R-induced cell death. Although further investigations on the cellular mechanisms involved in sAC-induced cardioprotection are necessary, the intra-mitochondrial sAC appears to play a major role in the recovery of cellular homeostasis after CA/R, representing a possible target for the development of new strategies to protect the heart against reperfusion injury. Interestingly, recent works have shown the presence of PDE2 in the mitochondrial matrix, and demonstrate its role in controlling cAMP generation by inhibiting sAC in liver and cardiac cells mitochondria (Acin-Perez et al., 2011; Pozdniakova S et al., 2016, Keystone abstract), thus providing further strategies to potentiate cAMP synthesis in mitochondria and to prevent injury in the clinical setting.
5 References


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