Mechanism of cGMP-mediated protection in a cellular model of myocardial reperfusion injury


Institute of Physiology, Justus-Liebig-University, Aulweg 129, D-35392 Giessen, Germany

Received 2 July 2004; received in revised form 16 December 2004; accepted 3 January 2005
Available online 30 January 2005
Time for primary review 21 days

Abstract

Objective: Reperfusion injury of the myocardium is characterised by development of cardiomyocyte hypercontracture. Previous studies have shown that cGMP-mediated stimuli protect against reperfusion injury, but the cellular mechanism is still unknown.

Methods: To simulate ischemia/reperfusion, adult rat cardiomyocytes were incubated anoxically (pH 6.4) and then reoxygenated (pH 7.4). Cytosolic calcium ([Ca\textsuperscript{2+}]\textsubscript{i} (fura-2 ratio), pH (BCECF ratio), cell length, and phospholamban phosphorylation were analysed. Under simulated ischemia cardiomyocytes develop [Ca\textsuperscript{2+}]\textsubscript{i} overload. When reoxygenated they rapidly undergo hypercontracture, triggered by oscillations of [Ca\textsuperscript{2+}]\textsubscript{i}. We investigated whether cGMP-mediated stimuli can modulate [Ca\textsuperscript{2+}]\textsubscript{i} or pH recovery and whether this contributes to their protective effect. Membrane-permeable cGMP analogues, 8-bromo-cGMP (1 mmol/L) or 8-pCPT-cGMP (10 \textmu mol/L), or a receptor-mediated activator of particulate guanylyl cyclase, urodilatin (1 \textmu mol/L), were applied.

Results: The investigated stimuli protect against reoxygenation-induced hypercontracture (cell length as percent of end-ischemic length; control: 68 \pm 1.6; 8-bromo-cGMP: 88 \pm 1.5*; 8-pCPT-cGMP: 84 \pm 2.9*; urodilatin: 87 \pm 1.1*; n=24; *p<0.05). Recovery from [Ca\textsuperscript{2+}]\textsubscript{i} overload after 2 min reoxygenation [fura-2 ratio (a.u.); control: 1.43 \pm 0.15; 8-bromo-cGMP: 1.86 \pm 0.15*; 8-pCPT-cGMP: 1.92 \pm 0.19*; urodilatin: 1.93 \pm 0.24*; n=25; *p<0.05] was accelerated, and the frequency of [Ca\textsuperscript{2+}]\textsubscript{i} oscillations (min/C\textsubscript{0}1) was significantly reduced (control: 49 \pm 5.0 min \textsuperscript{-1}; 8-bromo-cGMP: 18 \pm 3.5* min \textsuperscript{-1}; 8-pCPT-cGMP: 18 \pm 4.5* min \textsuperscript{-1}; urodilatin: 16 \pm 4.1* min \textsuperscript{-1}; n=24; *p<0.05). cGMP-mediated stimuli increased sarcoplasmic Ca\textsuperscript{2+} sequestration (caffeine-releasable Ca\textsuperscript{2+} pool: 2–3 fold increase vs. control). Inhibition of sarcoplasmic Ca\textsuperscript{2+}-ATPase (SERCA) by thapsigargin (150 nmol/L) or of protein kinase G with KT-5823 (1 \textmu mol/L) abolished the effect of these stimuli on [Ca\textsuperscript{2+}]\textsubscript{i} recovery. The investigated stimuli significantly enhanced phospholamban phosphorylation.

Conclusions: We conclude that cGMP-dependent signals activate SERCA via a protein kinase G-dependent phosphorylation of phospholamban. The increase in SERCA activity seems to reduce peak [Ca\textsuperscript{2+}]\textsubscript{i} and [Ca\textsuperscript{2+}]\textsubscript{i} oscillation during reoxygenation and to attenuate the excessive activation of the contractile machinery that otherwise leads to the development of hypercontracture.

Keywords: Reperfusion injury; Cardiomyocytes; Calcium; cGMP; Sarcoplasmic reticulum

1. Introduction

Re-supply of oxygen to the myocardium after extended periods of ischemia can rapidly aggravate the already existing injury. In several previous publications we and others have shown that acute reperfusion injury in isolated cardiomyocytes is characterised by the sudden development of hypercontracture [1–3]. In reoxygenated cardiac cells, re-supply of energy at still elevated Ca\textsuperscript{2+} levels causes this excessive contractile activation. Hypercontracture is characterised by an abrupt and extreme reduction of cell length during the initial minutes of reoxygenation. In reperfused myocardium it leads to the typical histological pattern of
contraction band necrosis [4,5]. It has been demonstrated before that, mechanisms which prevent reoxygenation-induced hypercontracture in isolated cardiomyocytes can also protect the myocardium in vivo against reperfusion-induced necrosis, i.e. acute lethal reperfusion injury [2,6–9].

Protective effects against myocardial reperfusion injury by cGMP-mediated stimuli have been described in different experimental models. For example, activation of the soluble guanylyl cyclase by NO donors or substrates of the NO synthase, activation of the particulate guanylyl cyclase with ANP or urodilatin, or inhibition of enzymes that hydrolyze cGMP, such as phosphodiesterase type 5, have been shown to protect myocardium against reperfusion injury [6,8,10–12]. Among these therapeutic strategies the use of urodilatin is of particular interest. It was shown in vivo in a pig heart model that urodilatin, applied at low doses, reduces infarct size without adverse hemodynamic side effects [8]. Urodilatin is a member of the atrial natriuretic peptide family that is naturally generated in the distal tubuli of the kidney. In systemic application, urodilatin has a longer plasma half life and fewer hemodynamic side effects compared with ANP [13]. The mechanism of protection provided via cGMP signaling in reoxygenated myocardial cells is still unknown. In isolated cardiomyocytes cGMP signaling can reduce the myofibrillar Ca2+ sensitivity [14]. This may confer protection during the early phase of reoxygenation. We showed before that a temporary inhibition of the contractile apparatus at the time of reperfusion protects cardiomyocytes, whether isolated or in situ, against reperfusion injury [9]. The Ca2+ desensitising effect of cGMP signals on the contractile apparatus, however, is relatively weak. It is also conceivable that cGMP acts through other mechanisms to provide protection. Signals arising from cGMP may, e.g., alter cytosolic Ca2+ or pH control in cardiomyocytes during the critical, early phase of reoxygenation.

The aim of the present study was to investigate whether application of cGMP-mediated stimuli can modulate [Ca2+]i or pH recovery in reoxygenated cardiomyocytes and whether this contributes to their protective effect. Applications of the cell-permeable cGMP analogue 8-bromo-cGMP, the specific activator of cGMP-dependent protein kinase 8-pCPT-cGMP, and urodilatin were compared. The study was performed with isolated cardiomyocytes from adult rats. These were exposed to anoxia in medium with pH 6.4, to simulate ischemia, and subsequently reoxygenated in medium with pH 7.4, to simulate reperfusion. This experimental model has been described previously in great detail [1–3].

2. Material and methods

2.1. Isolation of cardiomyocytes

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85–23 1985). Ventricular cardiomyocytes were isolated from 200–250 g adult male Wistar rats as described in detail previously [15].

2.2. Ca2+, pH and cell length measurements

To measure cytosolic Ca2+ or H+ concentrations, cardiomyocytes were loaded at 37 °C with fura-2 or BCECF, respectively as described in our previous studies [1–3]. Cover-slips with loaded cells were introduced into a gas-tight, temperature-controlled (37 °C), transparent perfusion chamber positioned in the light path of an inverted microscope. Alternating excitation of the fluorescent dye at wavelengths of 340/380 nm for fura-2 and 440/490 nm for BCECF was performed with an AR-Cation Measurement System adapted to the microscope. Light emitted (500–520 nm for fura-2 and 520–560 nm for BCECF) from an area of 10×10 μm within a single fluorescent cell was collected by the photomultiplier of the system. Simultaneously, the cell’s microscopic image was recorded with a video camera. Changes in cell length were determined from these recordings. The fura-2 ratio and BCECF ratio were calibrated as described previously [3,16,17,18]. When fura-2 ratio data are presented, these are expressed as arbitrary units with the resting values set at 1. Time course of fura-2 ratio during reoxygenation is presented as systolic (upper level of Ca2+ oscillations) and diastolic values (lower level of Ca2+ oscillations).

2.3. Media

The HEPES-buffered medium contained (mmol/L): 125.0 NaCl, 2.6 KCl, 1.2 KH2PO4, 1.2 MgSO4, 1.0 CaCl2, and 25.0 HEPES. For normoxia, medium pHo was adjusted to 7.4 and 2.5 mmol/L glucose was added. For anoxia, medium was autoclaved and gassed with 100% N2, glucose was omitted, and then pHs was adjusted to 6.4. 8-pCPT-cGMP (10 μmol/L), 8-bromo-cGMP (1 mmol/L), or urodilatin (1 μmol/L) were added to media 5 min before reoxygenation, whereas thapsigargin (150 mmol/L) and KT 5823 (1 μmol/L) were added 15 min before reoxygenation. For Mn2+ quench experiments, MnCl2 (100 μmol/L) was added at the onset of reoxygenation. To assess the Ca2+ load of sarcoplasmic reticulum, caffeine (20 mmol/L) was applied at the end of reoxygenation.

2.4. Determination of phospholamban phosphorylation

Phospholamban phosphorylation was determined as described previously in detail [19]. For Western blotting cardiomyocytes were treated with lysis buffer after 5 min of reoxygenation in the presence or absence of 8-bromo-cGMP or urodilatin. Protein extracts from cardiomyocytes were prepared and loaded (75–80 μg) onto an 18.5% (w/v) SDS-polyacrylamide gel as described by Laemmli [20]. After electrophoresis; proteins were transferred by semi-dry...
blotting onto PVDF membranes. Sheets were saturated with 3% (w/v) bovine serum albumin for 1 h and incubated with a phospholamban antibody (anti-PLB) or a phosphospecific phospholamban antibody (anti-pPLB, Ser16, 0.5 μg/mL). After washing, sheets were incubated for 2 h with an alkaline phosphatase-labeled anti-rabbit IgG antibody (0.5 μg/mL). Bands were finally visualised by alkaline phosphatase reactivity using 5-bromo-4-chlor-3-indolyl phosphate and nitro blue tetrazolium. Total phospholamban and phosphorylated phospholamban was quantified densitometrically. Statistical analysis was performed on the data of phosphorylated phospholamban vs. total phospholamban.

2.5. Experimental protocols

2.5.1. Anoxia–reoxygenation experiments

After 10 min normoxic perfusion, cardiomyocytes were superfused (0.5 mL/min) with the anoxic glucose-free medium (pH 6.4) for 60–70 min and then reoxygenated for 15 min with normoxic medium. Cardiomyocytes were not stimulated.

2.6. Statistics

Data are given as mean values±S.E.M. from n individual cells investigated in separate experiments. Statistical comparisons were performed by one-way ANOVA and use of the Student–Newman–Keuls test for post hoc analysis. Differences with p<0.05 were regarded as statistically significant.

3. Results

3.1. cGMP-mediated protection against reoxygenation-induced hypercontracture

During reoxygenation, isolated cardiomyocytes develop irreversible hypercontracture. Fig. 1A shows the time course of changes in cell length (presented in percentage of end-ischemic cell length) during 15 min reoxygenation under control conditions and in the presence of 8-bromo-cGMP (1 mmol/L). The presence of the cell-permeable cGMP analogue reduced significantly the hypercontracture development.

In Fig. 1B, the extent of cell shortening after 15 min reoxygenation is compared for cells reoxygenated in the presence of 8-bromo-cGMP (1 mmol/L), the specific PKG activator 8-pCPT-cGMP (10 μmol/L), and the receptor agonist urodilatin (1 μmol/L). At the chosen concentrations, the effects of all three treatments were comparable.

3.2. Cytosolic Ca²⁺ recovery

The ratio of fura-2 fluorescence was monitored to evaluate changes in cytosolic Ca²⁺ concentration during simulated ischemia and reoxygenation. During simulated ischemia, isolated cardiomyocytes developed cytosolic Ca²⁺-overload. According to the calibration protocol, the initial ratio of 1 in normoxic cells corresponds to [Ca²⁺], of 72 nmol/L, the end-anoxic fura-2 ratio of 4 corresponds to [Ca²⁺], of 1.9 μmol/L. The end-anoxic value represents severe Ca²⁺ overload. When cells were reoxygenated in medium with pH 7.4, the fura-2 ratio declined to the preanoxic value within 15 min. Fig. 2 shows representative traces of the fura-2 ratio during anoxia and reoxygenation under control conditions and in the presence of 8-bromo-cGMP.

As shown in Fig. 3A the administration of 8-bromo-cGMP accelerates both systolic and diastolic [Ca²⁺],
recovery, especially during the first 5 min. In Fig. 3B the changes of systolic and diastolic fura-2 recovery during the first 2 min of reoxygenation are compared for 8-bromo-cGMP, 8-pCPT-cGMP, and urodilatin. All described interventions accelerate the rate of both systolic and diastolic Ca\(^{2+}\) recovery. At the chosen concentrations, the effects of all three treatments were comparable, i.e., they equally accelerated the decline of systolic and diastolic cytosolic Ca\(^{2+}\) concentration.

Concomitantly with the Ca\(^{2+}\) recovery in reoxygenated cardiomyocytes, transient Ca\(^{2+}\) oscillations were found to occur [1–3]. In the present experiments, these oscillations reached a maximum at the second minute of reoxygenation. Fig. 4A shows the mean value of the oscillation frequency under control conditions and in the presence of 8-bromo-cGMP during the first 15 min of reoxygenation. The rapid Ca\(^{2+}\) oscillations occurring under control conditions were reduced under 8-bromo-cGMP treatment. In Fig. 4B, the 2-min values of oscillation frequencies are compared for 8-bromo-cGMP, 8-pCPT-cGMP, and urodilatin. The three treatments attenuate greatly the Ca\(^{2+}\) oscillations. At the chosen concentrations, the effects are comparable.

3.3. Sarcolemmal Ca\(^{2+}\) influx

The mechanism by which cGMP-mediated stimuli induce the observed acceleration in Ca\(^{2+}\) recovery was investigated for 8-bromo-cGMP. First, we tested the influence of 8-bromo-cGMP on the influx of extracellular Ca\(^{2+}\). As published before, Ca\(^{2+}\) influx during the early phase of reoxygenation can play an important role as a trigger for spontaneous Ca\(^{2+}\) oscillations and subsequent hypercontracture in cardiomyocytes [2]. Mn\(^{2+}\)-quench experiments were performed to quantify the Ca\(^{2+}\) influx.

In these experiments the 340-, 380- and 360-nm fluorescence were recorded in fura-2 loaded cells. The ratio of 340- and 380-nm fluorescence reflects the change of cytosolic Ca\(^{2+}\) concentration. The 360-nm fluorescence represents the isosbestic point of the fura-2 excitation spectrum and does not change with respect to the Ca\(^{2+}\) binding to the dye but is sensitive to Mn\(^{2+}\). As a bivalent cation, Mn\(^{2+}\) uses the same entry mechanism through the plasmalema of cells as Ca\(^{2+}\). The influx of Mn\(^{2+}\) in fura-2 loaded cells quenches the 360-nm fluorescence and the quench rate therefore indicates the influx rate of Ca\(^{2+}\). As shown in Fig. 5, 8-bromo-cGMP did not influence the quench rate, indicating that this cGMP signal does not influence the sarcolemmal Ca\(^{2+}\) influx.

The Na\(^{+}/Ca\(^{2+}\)-exchanger (NCX) plays a substantial role in the control of cytosolic Ca\(^{2+}\) in cardiac cells. In
reoxygenated cardiomyocytes an initial operation of this
exchanger in its “reverse mode” may induce additional Ca2+
influx and thereby aggravate the existing disorder of cation
homeostasis [2]. Later, its operation in “forward mode”
represents the key mechanism for the eventual removal of
Ca2+ from the cytosol. We showed previously that both
modes of operation may occur in sequential order in
cardiomyocytes during the early phase of reoxygenation
[2,3]. Considering these facts we applied another protocol to
investigate the influence of 8-bromo-cGMP on the forward
and reverse mode of the Na+/Ca2+-exchanger. Cardiomyo-
cytes were first incubated in Na+-free medium and
subsequently reincubated in medium containing Na+. This
treatment forces the Na+/Ca2+-exchanger first into a reverse,
then into a forward mode of operation. Incubation of the
cardiomyocytes under Na+-free conditions resulted in an
increase in the cytosolic fura-2 ratio (from 1.05±0.08 to
5.90±0.09*, *p<0.05, n=28), mediated by reverse mode
activation of the Na+/Ca2+-exchanger. Subsequent re-incu-
bation in medium containing Na+ led to a complete recovery
from Ca2+ overload via activation of forward mode of the
Na+/Ca2+-exchanger. The fura-2 ratio returned to 1.10±0.10
within 15 min. In this protocol, pre-incubation with 8-
bromo-cGMP influenced neither the reverse mode nor the
forward mode of the Na+/Ca2+-exchanger. The fura-2 ratio
in the presence of 8-bromo-cGMP under Na+-free con-
ditions was 5.82±0.14; after re-addition of Na+ it was
1.15±0.20 (n.s. vs. control; n=25).

3.4. The role of the sarcoplasmic reticulum

In order to investigate whether 8-bromo-cGMP acceler-
ates Ca2+ recovery by increasing the Ca2+ uptake into the
sarcoplasmic reticulum, cardiomyocytes were reoxygenated
with thapsigargin, an inhibitor of the SR Ca2+-ATPase, in
the presence and absence of 8-bromo-cGMP. Fig. 6 shows
that inhibition of sarcoplasmic Ca2+-ATPase by thapsigar-
gin abolished the accelerating effect of 8-bromo-cGMP on
cytosolic Ca2+ recovery. This indicates that the accelerating
effect of cGMP on Ca2+ recovery depends on SR function.
As this could be due to a cGMP-dependent activation of
the SR Ca2+-ATPase, we analysed the phosphorylation
state of its regulatory protein, phospholamban. We found
that in the presence of cGMP-mediated stimuli during the
reperfusion period the phosphorylation of phospholamban is
increased. This indicates an activation of the SR Ca2+-
ATPase. The upper panel of Fig. 7 shows a representative
Western blot. When 8-bromo-cGMP, 8pCPT-cGMP or
urodilatin were applied during reoxygenation, phospho-
lamban phosphorylation was significantly higher than in
reoxygenated controls.

![Graph A](image1)

Fig. 4. (A) Time course of oscillation frequency of the fura-2 ratio signal under control conditions (●) and in the presence of 8-bromo-cGMP (○). Data are given as mean values±S.E.M.; n=24 cells from 6 different preparations; *p<0.05. (B) Oscillation frequency after 2 min of reoxy-
genation under control conditions, in the presence of 8-bromo-cGMP, 8-
pCPT-cGMP, or urodilatin. Data are given as mean values±S.E.M.; n=24
cells from 6 different preparations; *p<0.05.

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

![Graph E](image5)

![Graph F](image6)

![Graph G](image7)

![Graph H](image8)

![Graph I](image9)

![Graph J](image10)

![Graph K](image11)

![Graph L](image12)

![Graph M](image13)

![Graph N](image14)

![Graph O](image15)

![Graph P](image16)

![Graph Q](image17)

![Graph R](image18)

![Graph S](image19)

![Graph T](image20)

![Graph U](image21)

![Graph V](image22)

![Graph W](image23)

![Graph X](image24)

![Graph Y](image25)

![Graph Z](image26)

![Graph AA](image27)

![Graph AB](image28)

![Graph AC](image29)

![Graph AD](image30)

![Graph AE](image31)

![Graph AF](image32)

![Graph AG](image33)

![Graph AH](image34)

![Graph AI](image35)

![Graph AJ](image36)

![Graph AK](image37)

![Graph AL](image38)

![Graph AM](image39)

![Graph AN](image40)

![Graph AO](image41)

![Graph AP](image42)

![Graph AQ](image43)

![Graph AR](image44)

![Graph AS](image45)

![Graph AT](image46)

![Graph AU](image47)

![Graph AV](image48)

![Graph AW](image49)

![Graph AX](image50)

![Graph AY](image51)

![Graph AZ](image52)

![Graph BA](image53)

![Graph BB](image54)

![Graph BC](image55)

![Graph BD](image56)

![Graph BE](image57)

![Graph BF](image58)

![Graph BG](image59)

![Graph BH](image60)

![Graph BI](image61)

![Graph BJ](image62)

![Graph BK](image63)

![Graph BL](image64)

![Graph BM](image65)

![Graph BN](image66)

![Graph BO](image67)

![Graph BP](image68)

![Graph BQ](image69)

![Graph BR](image70)

![Graph BS](image71)

![Graph BT](image72)

![Graph BU](image73)

![Graph BV](image74)

![Graph BW](image75)

![Graph BX](image76)

![Graph BY](image77)

![Graph BZ](image78)
The lower panel of the figure shows the densitometrical analysis of the phosphorylation state of phospholamban under normoxic conditions, at the end of anoxia and after 5 min of reoxygenation in the presence or absence of 8-bromo-cGMP, 8-pCPT-cGMP or urodilatin. Presence of protein kinase G inhibitor, KT 5283, attenuated significantly the increase of phospholamban phosphorylation under stimulation with 8-bromo-cGMP, 8-pCPT-cGMP or urodilatin.

Increased SR-Ca$^{2+}$ uptake may lead to an increase in Ca$^{2+}$ sequestration into the SR. To test this hypothesis, we applied caffeine to cardiomyocytes at the end of reoxygenation. Fig. 8A shows a representative experiment comparing the application of caffeine to cardiomyocytes at the end of reoxygenation in presence or absence of urodilatin. Caffeine-induced rise of cytosolic Ca$^{2+}$, indicating the Ca$^{2+}$ depletion of the SR, is higher in the cell reoxygenated in presence of urodilatin. This experiment was also repeated under 8-bromo-cGMP and 8-pCPT-cGMP treatment. The statistical analysis in Fig. 8B shows that the caffeine-induced rise of cytosolic Ca$^{2+}$ in reoxygenated cardiomyocytes treated with urodilatin, 8-bromo-cGMP or 8-pCPT-cGMP is significantly higher (five to six-fold) than under control conditions (two-fold). These data show that cGMP-mediated stimuli can indeed augment sequestration of Ca$^{2+}$ into the SR in reoxygenated cardiomyocytes.

3.5. Involvement of PKG

In order to test whether the change in cytosolic Ca$^{2+}$ recovery in presence of cGMP-mediated stimuli is dependent on protein kinase G, we applied the inhibitor KT 5283 (10 μmol/L) 10 min before and during reoxygenation in presence of 8-bromo-cGMP, 8-pCPT-cGMP or urodilatin. In
presence of this inhibitor, cGMP-mediated stimuli failed to accelerate the cytosolic Ca\textsuperscript{2+} recovery or to reduce the frequency of Ca\textsuperscript{2+} oscillations. The changes of diastolic fura-2 ratio during the first 2 min of reoxygenation were 1.20±0.15 under control conditions, 1.15±0.09 for 8-bromo-cGMP+KT, 1.23±0.14 for 8-pCPT-cGMP+KT and 1.10±0.11 for urodilatin+KT (n.s. vs. control, n=24). The changes of systolic fura-2 ratio during the first 2 min of reoxygenation were 0.60±0.04 under control conditions, 0.65±0.11 for 8-bromo-cGMP+KT, 0.63±0.08 for 8-pCPT-cGMP+KT and 0.58±0.15 for urodilatin+KT (n.s. vs. control, n=24). The 2-min values of Ca\textsuperscript{2+} oscillation frequencies were 47±7 min\textsuperscript{-1} under control conditions, 45±8 min\textsuperscript{-1} for 8-bromo-cGMP+KT, 51±5 min\textsuperscript{-1} for 8-pCPT-cGMP+KT and 47±9 min\textsuperscript{-1} for urodilatin+KT (n.s. vs. control, n=24). These results indicate the effects of cGMP-mediated stimuli on cytosolic Ca\textsuperscript{2+} kinetics are mediated by protein kinase G.

### 3.6. Effects on cytosolic pH recovery

Under normoxic control conditions, the pH\textsubscript{i} of cardiomyocytes was 7.10±0.09. Superfusion of cardiomyocytes with anoxic medium at pH\textsubscript{o} 6.4 led to a pronounced acidification of the cytosol. After 70 min, the pH\textsubscript{i} was 6.40±0.07\textsuperscript{*} (*p<0.05 vs. normoxic conditions; n=25). During reoxygenation the intracellular pH recovered within 15 min to the pre-anoxic control value. As the following data show, neither 8-bromo-cGMP nor 8-pCPT-cGMP or urodilatin influenced the pH\textsubscript{i} recovery: after 15 min of reoxygenation under control conditions, the pH\textsubscript{i} was 7.20±0.12 (n=25). At the same time point, the pH\textsubscript{i} was 7.24±0.08 in the presence of 8-bromo-cGMP (n.s. vs. control; n=25). In the presence of 8-pCPT-cGMP, the pH\textsubscript{i} was 7.21±0.13, (n.s. vs. control, n=25). In the presence of urodilatin, the pH\textsubscript{i} was 7.18±0.06 (n.s. vs. control; n=25). Thus cGMP-mediated stimuli did not change pH\textsubscript{i} recovery in reoxygenated cardiomyocytes.

### 4. Discussion

The aim of the present study was to investigate on the cellular level the mechanism by which cGMP-mediated stimuli can protect cardiomyocytes against reoxygenation-induced hypercontracture. The focus was on the control of cellular Ca\textsuperscript{2+} as it has been shown that re-supply of energy in Ca\textsuperscript{2+} overloaded cardiomyocytes in combination with Ca\textsuperscript{2+} oscillations trigger their hypercontracture during the initial phase of reoxygenation. The main result of this study is that cGMP-mediated stimuli interfere with this mechanism by acceleration of cytosolic Ca\textsuperscript{2+} recovery and attenuation of Ca\textsuperscript{2+} oscillations.

The three investigated stimuli conferred similar protective effects. 8-bromo-cGMP is known to mimic most intracellular effects of cGMP. The cGMP analogue 8-pCPT-cGMP is a specific activator of protein kinase G. Urodilatin was used as a receptor-mediated activator of membrane-bound guanylyl cyclase. At the chosen concentration, it had comparable effects to the cGMP analogues on cytosolic Ca\textsuperscript{2+} kinetics. These effects were abolished by KT5283, a specific inhibitor of protein kinase G.

Previous studies on reoxygenation-induced hypercontracture of cardiomyocytes have analysed the causal role of Ca\textsuperscript{2+} oscillations for hypercontracture in detail [1–3]. It is known from these studies that it is sufficient to suppress the oscillatory Ca\textsuperscript{2+} rise during the first minutes of reoxygenation to achieve protection against development of hypercontracture. These Ca\textsuperscript{2+} oscillations are provoked by the Ca\textsuperscript{2+} overload, persisting from the preceding anoxia or ischemia, in a cell in which the resumption of oxidative energy production reactivates Ca\textsuperscript{2+} uptake by the SR. Oscillations of cytosolic Ca\textsuperscript{2+} result from repetitive episodes of uptake of cytosolic Ca\textsuperscript{2+} into the SR and subsequent spontaneous Ca\textsuperscript{2+} release from the then Ca\textsuperscript{2+}-overloaded.
SR. Ca2+ oscillations can be accelerated in frequency and prolonged in duration when a net Ca2+ influx is still ongoing at the beginning of reoxygenation. We showed previously that this may occur due to an initial reverse-mode operation of the sarcolemmal Na+/Ca2+-exchanger [20]. Ca2+ oscillations normally end spontaneously once the trans-sarcolemmal Ca2+ extrusion has sufficiently reduced the total cellular Ca2+ overload. Net outward transport of Ca2+ from the cytosol depends on re-establishment of a forward mode operation of the Na+/Ca2+-exchanger, which occurs when a trans-sarcolemmal Na+ gradient has been restored and the sarcolemma is repolarised.

In the present study we found for all cGMP-mediated stimuli alike that these enhance the Ca2+ clearing from the cytosol and reduce Ca2+ oscillations. This change in Ca2+ kinetics did not result from a reduction in Ca2+ influx, as shown by the Mn2+ quench experiments. It seems also unlikely that the change is due to enhanced Ca2+ efflux, as we showed that cGMP signals have no effect on the main Ca2+ extruder, i.e., the Na+/Ca2+-exchanger. Instead, the changes in initial cytosolic Ca2+ kinetics can be attributed to an altered SR function in the reoxygenated cells. This can be inferred from the following findings: First, thapsigargin experiments showed that in presence of a blocker of SR Ca2+-ATPase the differences in cytosolic Ca2+ kinetics were abolished. Second, the regulatory protein of the SR Ca2+-ATPase, phospholamban, was phosphorylated to a larger extent in presence of cGMP-mediated stimuli in reoxygenated cells. This coincides with previous study showing that cGMP-mediated stimuli increase phosphorylation of phospholamban in cardiac myocytes [21]. Note that a phosphorylated phospholamban allows for a greater activity of the SR Ca2+-pump [22–24]. Third, the caffeine-releasable pool of Ca2+ in cells reoxygenated in presence of cGMP-mediated stimuli was enlarged. Based on these data in reoxygenated cardiomyocytes, the following mechanism can be proposed: cGMP-dependent signals activate SR Ca2+-ATPase via a protein kinase G-dependent phosphorylation of phospholamban. This increase in SR Ca2+-ATPase activity enlarges the pool of Ca2+ stored within the sarcoplasmic reticulum. This supports an early clearing of excess Ca2+ from the cytosol and reduces the oscillatory Ca2+ rise.

The reduction of the peak cytosolic Ca2+ concentrations during the early phase of reoxygenation attenuates the activation of the contractile machinery, otherwise developing under these reoxygenation conditions. cGMP-mediated stimuli did not affect the changes in intracellular pH occurring during the ischemia–reoxygenation protocol. This was worth testing since control of cytosolic Ca2+ and contractile activation in the reoxygenated cell are distinctly pH dependent.

The basic principle of cardioprotection identified here may give rise to new strategies aimed at protection against reperfusion injury in the clinical setting. The use of agonists that activate the particulate guanylyl cyclase such as urodilatin and other natriuretic peptides seems particularly promising. It has already been shown that urodilatin can be used for protection of the reperfused heart in vivo [18]. Compared to the use of NO donors, which activate the soluble guanylyl cyclase, this would appear advantageous since natriuretic peptides have only little effect on systemic hemodynamics and give no rise to toxic metabolites such as peroxynitrite [25,26].

Acknowledgments

The technical help of D. Schreiber, D. Miekley, G. Schade, A. Stapler, and S. Kechter is gratefully acknowledged. This work is part of a thesis submitted by Y. Abdallah in fulfillment of the requirements for the degree of Doctor of Medicine at the Justus-Liebig-Universität, Gießen.

References


