Cardioprotective effects of KB-R7943: a novel inhibitor of the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchanger

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Ladilov, Y., S. Haflner, C. Balser-Schäfer, H. Maxeiner, and H. M. Piper. Cardioprotective effects of KB-R7943: a novel inhibitor of the reverse mode of Na\(^+\)/Ca\(^{2+}\) exchanger. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1868–H1876, 1999.—The novel inhibitor of the reverse mode of the Na\(^+\)/Ca\(^{2+}\) exchanger (NCE) KB-R7943 (KB) was tested in isolated rat cardiomyocytes exposed to 80 min of simulated ischemia [substrate-free anoxia, extracellular pH (pH\(_{o}\)) of 6.4] and 15 min of reoxygenation (pH\(_{o}\) 7.4). At pH\(_{o}\) 6.4, 20 µmol/l KB was required for complete inhibition of the reverse mode of NCE. Treatment with 20 µmol/l KB only during anoxia did not influence the onset of rigor contracture and intracellular pH (pHi) (monitored with 2,7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein) but significantly reduced the cytosolic accumulation of Ca\(^{2+}\) (monitored with fura 2) and Na\(^+\) (monitored with sodium-binding benzofuran isophthalate). During reoxygenation, cardiomyocytes developed hypercontracture. This was significantly reduced by anoxic KB treatment. To investigate this protection against reoxygenation-induced injury in the whole heart, we exposed Langendorff-perfused rat hearts to 110 min of anoxia (pH\(_{o}\) 6.4) and 50 min of reoxygenation (pH\(_{o}\) 7.4). Application of 20 µmol/l KB during anoxia significantly reduced the reoxygenation-induced enzyme release. We conclude that KB offers significant protection of cardiomyocytes against Ca\(^{2+}\) and Na\(^+\) overload during anoxia and hypercontracture or enzyme release on reoxygenation.

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ACCUMULATION of Ca\(^{2+}\) in the cytosol during ischemia is an important factor to induce ischemic and reperfusion injury of myocardial cells (14, 26). The mechanisms responsible for ischemic Ca\(^{2+}\) overload are still a matter of debate. It has been supposed that the Na\(^+\)/Ca\(^{2+}\) exchanger (NCE) represents an important route by which Ca\(^{2+}\) enters the ischemic cardiomyocyte (5, 16, 27, 32). Under ischemic conditions, the decrease of the transmembrane Na\(^+\) gradient and depolarization of the sarcolemma led to a reduction in the net-outward transport of Ca\(^{2+}\) via the forward mode of NCE and may even favor a net Ca\(^{2+}\) influx via the reverse mode of NCE. One may expect, therefore, that inhibition of the reverse mode of NCE would provide protection of ischemic cells against Ca\(^{2+}\) overload.

The evidence supporting the role of NCE for ischemic Ca\(^{2+}\) overload is based on experiments in which the transsarcolemmal Na\(^+\) gradient was manipulated (5) or agents with little specificity, like amiloride (16) or dimethylthiourea (32), were used. Recently, KB-R7943 (KB), a novel inhibitor of the sarcolemmal NCE, has been introduced (9). It has been shown that KB inhibits preferentially the reverse mode of NCE with a lower potency for the forward mode of NCE and other ion transport systems, such as the Na\(^+\)/H\(^+\) exchanger, L-type Ca\(^{2+}\) channels, voltage-gated Na\(^+\) channels, and inward rectifier K\(^+\) channels (9, 31).

The aim of the present study was first to test, on the cellular level, the effects of KB on cytosolic ion homeostasis (Ca\(^{2+}\), Na\(^+\), and H\(^{+}\)) during ATP depletion. For this purpose, we used a previously characterized model of simulated ischemia [substrate-free anoxia in combination with low extracellular pH (pH\(_{o}\)) in isolated cardiomyocytes (11–13). Second, we also investigated whether inhibition of the reverse mode of NCE during anoxia provides protection in the early phase of reoxygenation. To research this idea, the effect of KB on reoxygenation-induced hypercontracture of cardiomyocytes, the main underlying mechanism of acute reoxygenation-induced severe cell injury (4, 24), was analyzed. Third, using an isolated perfused heart model, we investigated whether the intra-anoxic presence of KB reduces the extent of reoxygenation-induced enzyme release in the whole heart. We report here that treatment with KB during anoxia effectively inhibits Ca\(^{2+}\) overload and attenuates reoxygenation-induced injury of cardiomyocytes.

MATERIALS AND METHODS

Preparation of Isolated Cardiomyocytes

Ventricular heart muscle cells were isolated from 250- to 300-g adult male Wistar rats as described previously (20). Briefly, isolated hearts were subjected to a recirculating Langendorff perfusion with buffer containing 110.0 mmol/l NaCl, 2.6 mmol/l KCl, 1.2 mmol/l KH\(_{2}\)PO\(_{4}\), 1.2 mmol/l MgSO\(_{4}\), 25.0 mmol/l NaHCO\(_{3}\), 11.0 mmol/l glucose, 25.0 µmol/l CaCl\(_{2}\), and 300 mg/l collagenase (type I, Worthington), at 37°C (20). The buffer was continuously gassed with carbogen to obtain a pH of 7.4. After 30 min of perfusion, the ventricular tissue was minced with a chopper and incubated in perfusate until cell separation was completed. Cardiomyocytes were purified by differential centrifugation. After isolation was completed, cells were plated in medium 199 with 4% fetal calf serum on glass coverslips that had been preincubated overnight with 4% fetal calf serum. Four hours after plating had been completed, the coverslips were washed with medium 199. As a result of the wash, broken cells were removed, leaving a homogeneous population (>95%) of rod-shaped quiescent cardiomyocytes attached to the coverslip. Cells were used for investigations from 6 to 12 h after preparation. From each isolation two to three coverslips were used. On each coverslip, four to six cells were investigated. Only cells exhibiting a rod-shaped morphology and no signs of sarcolemmal blebbing...
were used for the experiments. These cells were found to have a low resting cytosolic Ca\(^{2+}\) concentration.

\[\text{Ca}^{2+}, \text{Mg}^{2+}, \text{Na}^+, \text{pH, and Cell Length Measurements}\]

To measure cytosolic \(\text{Ca}^{2+}\), \(\text{Mg}^{2+}\), \(\text{Na}^+\), or \(\text{H}^+\) concentrations, cardiomyocytes were loaded in medium 199 at 35°C for 30 min with acetoxymethyl esters of fura 2 (2.5 µmol/l), Mg-fura 2 (2.5 µmol/l), sodium-binding benzofuran isophthalate (SBFI, 5.0 µmol/l), or 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF, 1.5 µmol/l), respectively. After loading was completed, cells were washed twice with medium 199 to allow hydrolysis of the acetoxymethyl esters within the cell. The fluorescence from dye-loaded cells was 30–40 times higher than background fluorescence from unloaded cells.

The coverslip with loaded cells was introduced into a gas-tight, temperature-controlled (37°C) transparent perfusion chamber positioned in the light path of an inverted microscope (Diaphot TMD, Nikon, Düsseldorf, Germany). Alternating excitation of the fluorescent dye at wavelengths of 340 and 380 nm for fura 2 and SBFI, 335 and 375 nm for Mg-fura 2, or 450 and 490 nm for BCECF was performed with an AR-Cation Measurement System adapted to the microscope (Spex Industries, Grasbrunn, Germany). Emitted light (490–510 nm for fura 2, Mg-fura 2, and SBFI, and 520–560 nm for BCECF) from a 10 × 10 µm area within a single fluorescent cell was collected by the photomultiplier of the Spex system. The light signal was recorded and analyzed by an IBM PC/AT-based data analysis system (model DM3000CM, Spex Industries, Grasbrunn, Germany). To avoid the dye bleaching during long-lasting anoxia-reoxygenation experiments, excitation of fura 2, SBFI, and BCECF was performed only at indicated points not longer than 20 s.

Simultaneous to the measurement of the fluorescence, the cell microscopic image was recorded with a video camera and stored on tape. From these recordings, changes of the cell length before anoxia during reoxygenation in relation to cell length before anoxia

\[
\text{hypercontracture} = \frac{L_{R(15)} - L_{R(0)}}{L_N}
\]

where \(L_{R(0)}\) is cell length before reoxygenation, \(L_{R(15)}\) is cell length after 15 min of reoxygenation, and \(L_N\) is cell length before anoxia.

**Dye Compartmentation**

The loading protocols 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 as described previously (12). It was found that the fluorescent signal from intracellular stores did not exceed 10% for fura 2, 15% for SBFI, and 12% for BCECF compared with the signal from the intact cell. Furthermore, extent of dye compartmentation did not differ significantly between control cells and cells after anoxia and reoxygenation. For the purpose of the present study, therefore, correction of the data for this small extent of dye compartmentation seemed unnecessary.

**In Vivo Calibration of Fura 2, SBFI, and BCECF**

Because of the inherent problems with calibration of fura 2, Mg-fura 2, and SBFI ratios, data were generally expressed in arbitrary units of fluorescence ratio. Control (before anoxia) and end-anoxic values of the fura 2 signal were calibrated as described previously (12). SBFI ratio was calibrated according to Harootunian et al. (6), with 6 µmol/l gramicidin D and incubation in media containing various Na\(^+\) concentrations. Calibration of the BCECF ratio signal was performed according to Koop et al. (10), with 10 µg/ml nigericin, a K\(^+\)/H\(^+\) ionophore, and incubation media with various pH values.

**Enzyme Activities**

During heart perfusion, samples of the effluent were collected during 20 s at the indicated times. In these samples, the activity of lactate dehydrogenase (LDH) and creatine kinase (CK) was estimated using standard diagnostic kits (Sigma-Chemie) and measuring NADH fluorescence at 460 nm under illumination with light at 355 nm. The total tissue activity of enzymes was also measured in whole heart homogenates \((n = 5)\) prepared in 100 mmol/l phosphate buffer \((\text{pH} 7.4)\) with 1% Triton X-100. Enzyme activity was expressed in units related to the dry weight of the whole heart.

**Media**

Isolated cardiomyocytes incubated in perfusion chamber (0.5 ml filling volume) were superfused at a flow rate of 0.6 ml/min with modified Tyrode solution containing (in mmol/l) 140.0 NaCl, 2.6 KCl, 1.2 KH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 1.0 CaCl\(_2\), 5.0 glucose, and 25.0 HEPES; \(\text{pH}\) was 7.4 at 37°C. Normoxic medium was equilibrated with air. Medium was made anoxic by autoclaving as described previously (1). The anoxic medium was glucose free and equilibrated before and during experiment with 100% \(\text{N}_2\).

**Experimental Protocols**

**Protocol A:** Na\(^+\) withdrawal in isolated cardiomyocytes. To estimate the capacity of KB to inhibit the reverse mode of NCE, the following protocol was applied. Under normoxic conditions, cardiomyocytes were pretreated for 1) 30 min with 150 nmol/l thapsigargin to inhibit the Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum (SR), 2) 5 min with 4 µmol/l ryanodine to inhibit Ca\(^{2+}\) release channels of SR and with 4 µmol/l HOE 642 to inhibit the sarcolemmal Na\(^+\)/H\(^+\) exchanger, and 3) 2 min with 300 µmol/l ouabain to inhibit Na\(^+\)-K\(^+\)-ATPase. The extracellular Na\(^+\) was then withdrawn in the presence of these substances. The osmolality of Na\(^+\)-free medium was corrected by the appropriate addition of N-methyl-d-glucamine. The rate of cytosolic Ca\(^{2+}\) accumulation was monitored with Ca\(^{2+}\)-fluorescent indicator fura 2 in control cells and cells treated with different concentrations of KB (Fig. 1).

Under anoxic conditions (see protocol B), cardiomyocytes were superfused with nominally Ca\(^{2+}\)-free anoxic medium \((\text{pH} 6.4)\) until they developed rigor contracture. Five minutes later 4 µmol/l ryanodine and 4 µmol/l HOE-642 were added. After an additional 5 min, extracellular Na\(^+\) was withdrawn in the presence of these substances.

**Protocol B:** Simulated ischemia-reperfusion in isolated cardiomyocytes. The protocol of simulated ischemia and reperfusion in single cardiomyocytes was established in our previous studies (9, 10). This protocol consisted of 80 min of anoxia at low \(\text{pH}_o\) 6.4 and 15 min of reoxygenation at \(\text{pH}_o\) 7.4. This protocol has been shown to produce rigor contracture, cytosolic Ca\(^{2+}\) overload \((\text{pCa} < 6)\), and acidosis \((\text{intracellular pH (pHi) 6.5})\) during anoxia and irreversible hypercontracture during reoxygenation, but it allows recovery of the cellular state of energy and ionic homeostasis on reoxygenation. Five groups of experiments were performed. In the control group,
the standard protocol of anoxia and reoxygenation was performed without modification. In the second group, anoxia was performed in the presence of 10 µmol/l nifedipine to inhibit L-type Ca\(^{2+}\) channels. In the third group, cardiomyocytes were pretreated for 30 min with 150 nmol/l thapsigargin to empty the SR and were treated during anoxia with 4 µmol/l ryanodine to inhibit the Ca\(^{2+}\) release channels of SR. In two other groups, cardiomyocytes were treated during anoxia with 10 or 20 µmol/l KB, an inhibitor of NCE. KB was washed out 10 min before reoxygenation. We found that this time was sufficient to abolish the inhibitory effect of KB in normoxic cardiomyocytes tested with a Na\(^{+}\) withdrawal protocol (data not shown).

Protocol C: Hypoxia reoxygenation in the whole heart.
Hearts from 250- to 300-g adult male Wistar rats were mounted on a Langendorff system in a temperature-controlled chamber (37°C) and perfused at a flow of 6 ml/min with Tyrode solution containing 11 mmol/l glucose. The chamber was filled with humidified air during normoxic perfusion and with 100% N\(_2\) during anoxic perfusion. In the control group, after the initial 30 min of equilibration with normoxic solution saturated with 100% O\(_2\) at pH 7.4, the hearts were perfused for 110 min with glucose-free anoxic solution saturated with 100% N\(_2\) at pH 6.4. During 50 min of reoxygenation, the hearts were resupplied with oxygen and glucose by changing back to the initial normoxic solution. In two other groups of experiments, KB at concentrations of 10 or 20 µmol/l was added to the anoxic perfusate and washed out 10 min before reoxygenation.

Materials
Medium 199 was purchased from Boehringer-Mannheim; fetal calf serum was from Gibco; acetoxymethyl esters of fura 2, Mg-fura 2, SBFI, and BCECF were from Paesel and Lorey; nifedipine and ouabain were from Sigma; ionomycin, nigericin, and ryanodine were from Calbiochem-Novabiochem; HOE-642 was a gift from Hoechst (Frankfurt/Main, Germany); KB-R7943 was a gift from Kanebo (Osaka, Japan). All other chemicals were from Merck and of highest purity.

### Statistics
Data are given as mean values ± SE. For each experimental protocol 20–50 individual cells were used, with not more than six cells from the same cell isolate. Statistical comparisons were performed by analysis of variance and by use of the Bonferroni test. Statistical significance was accepted when \( P < 0.05 \).

### RESULTS

Dose-Dependent Inhibition of Reverse Mode of NCE With KB

In previous studies, the inhibitory effect of KB on the reverse mode of NCE was tested at pH \( \leq 7.4 \) (8, 31). Under conditions of myocardial ischemia, pH\(_o\) is significantly lower. In the present study, medium with pH 6.4 was used to simulate ischemic acidosis. The inhibition of the reverse mode of NCE by KB was tested here in medium with pH 7.4 or pH 6.4. To intentionally activate the reverse mode of NCE, cardiomyocytes were superfused with Na\(^{+}\)-free medium. Under normoxic conditions, Na\(^{+}\) withdrawal led to the rapid rise of cytosolic Ca\(^{2+}\) monitored by the fura 2 ratio (Fig. 1A). At pH\(_o\) 7.4, the 10-min treatment of the cells with 10 µmol/l KB before and during Na\(^{+}\)-free superfusion was sufficient for complete inhibition of the reverse mode of NCE (Fig. 1B). At pH\(_o\) 6.4, however, 20 µmol/l KB was required to prevent activation of the reverse mode of NCE. The effect of KB disappeared completely within 10 min after removal of the drug from the incubation medium (data not shown).

### Dose-Dependent Inhibition of Reverse Mode of NCE With KB

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### Dose-Dependent Inhibition of Reverse Mode of NCE With KB

Under conditions of simulated ischemia, withdrawal of the extracellular Na\(^{+}\) also activated the reverse mode of NCE. The rise of the fura 2 ratio was significantly slower than that in normoxic cells (Fig. 1B). Treatment with 20 µmol/l KB abolished the rise of the fura 2 ratio.
Influence of Treatment With KB on Cytosolic Ca\(^{2+}\) Overload During Anoxia

Normoxic cardiomyocytes were quiescent and had cytosolic free Ca\(^{2+}\) of 54 ± 2 nmol/l. In the control group, superfusion of cardiomyocytes with anoxic glucose-free medium with pH 6.4 caused an accumulation of Ca\(^{2+}\) in the cytosol indicated by an increase in the fura 2 ratio (Fig. 2). Calibration of the fura 2 ratio showed that cytosolic Ca\(^{2+}\) concentration at the end of anoxia was 2.04 ± 0.06 µmol/l (n = 51). The accumulation of Ca\(^{2+}\) started ~30 min after the beginning of anoxia, directly after cells had developed rigor contracture. When cells were reoxygenated after 80 min of anoxia, the fura 2 ratio recovered to its initial level within the next 15 min.

Treatment with 10 µmol/l KB slightly but significantly reduced the cytosolic Ca\(^{2+}\) overload at the end of anoxia (1.41 ± 0.07 µmol/l, n = 44, P < 0.05 vs. control). Treatment with 20 µmol/l KB had a more pronounced effect, i.e., at the end of anoxia the cytosolic free Ca\(^{2+}\) was only 0.55 ± 0.03 µmol/l (n = 29, P < 0.05 vs. control and 10 µmol/l KB).

To emphasize that the reduction of anoxic Ca\(^{2+}\) overload with KB is indeed the result of the inhibition of NCE, the role of two other important routes for Ca\(^{2+}\) influx into the cytosol, i.e., the L-type Ca\(^{2+}\) channels of the sarcolemma and the Ca\(^{2+}\) release channels of the SR, was investigated. For this purpose, cardiomyocytes were exposed to anoxia in the presence of 10 µmol/l nifedipine or 4 µmol/l ryanodine. No differences were found in the degree of Ca\(^{2+}\) overload in comparison with control conditions (Fig. 2).

Changes in Cytosolic pH During Anoxia and Reoxygenation

Anoxia was combined with low pH\(_o\) (6.4) to simulate ischemic acidosis. As described previously (11), superfusion of cardiomyocytes with anoxic medium at pH\(_o\) 6.4 leads to a pronounced acidification of the cytosol. In the present study it reached pH\(_i\) 6.62 ± 0.03 (n = 17) after 80 min of anoxia in the control group (Fig. 4). When cells were treated with 20 µmol/l KB, intracellular acidosis developed to the same extent as without treatment (pH\(_i\) 6.66 ± 0.02, n = 16, P > 0.05 vs. control). Reoxygenation during 20 min in medium with pH 7.4 led to a recovery of pH\(_i\) to the initial level, with a similar rapidity under either experimental condition.

Influence of Treatment With KB on Time of Rigor Contracture and Cytosolic Mg\(^{2+}\) Concentration

During anoxia, cardiomyocytes shorten when their energy stores are depleted (2). This shortening is due to a rigor mechanism (rigor contracture). It is a rapid
process of cell length reduction by about one-third, completed within 30 s. Under control conditions, the onset of rigor shortening during anoxia occurred at 23.2 ± 1.3 min (n = 51). Treatment of the cells during anoxia with 10 or 20 µmol/l KB did not significantly influence the onset of rigor contracture (22.8 ± 1.1 min, n = 44, and 25.2 ± 1.5 min, n = 29). This indicates that KB does not influence the rate of ATP depletion. To confirm this, the fluorescent indicator Mg-fura 2 was used to monitor the rise in cytosolic free Mg2+ in anoxic cardiomyocytes. It has been shown previously that the concentration of Mg2+ in the cytosol increases during metabolic inhibition simultaneously with ATP degradation (25). We found that the Mg2+ concentration, indicated by the Mg-fura 2 ratio, started to rise during anoxia just a few minutes before rigor contracture and reached its plateau shortly after completion of rigor contracture (Fig. 5). Treatment with 20 µmol/l KB did not change this sequence of events (data not shown).

Influence of Treatment With KB on Reoxygenation-Induced Hypercontracture

As noted earlier in this paper, cell length was reduced during anoxia by about one-third as a result of rigor contracture. The degree of rigor contracture was not influenced by KB treatment. Reoxygenation of cells in the control group caused an additional rapid shortening, i.e., hypercontracture. The cell length was reduced by an additional 24.3 ± 0.9% (n = 51) relative to the normoxic cell length (Fig. 6). A slight but significant reduction of hypercontracture was observed in cells treated with 10 µmol/l KB (20.1 ± 1.1%, n = 44, P < 0.05). A more pronounced effect was observed when 20 µmol/l KB had been present during anoxia. Under this condition, the additional length reduction was only 9.2 ± 1.5% (n = 29, P < 0.01). Protection against reoxygenation-induced hypercontracture was thus dependent on the dose of KB.

Reduction of Reoxygenation-Induced Injury by Anoxic KB Treatment in Whole Heart

To investigate further whether the protection observed in isolated cardiomyocytes is relevant for the whole heart, the Langendorff-perfused heart model was used. The hearts were submitted to a protocol of anoxia-reoxygenation resulting in pronounced reoxygenation injury. After the perfusion was equilibrated for 30 min with normoxic Tyrode solution, the hearts were perfused for 110 min with glucose-free, anoxic solution at pHo 6.4. When reoxygenation after this period of anoxic perfusion was performed, a marked enzyme release was observed.

![Fig. 4. Time course of cytosolic pH during 80 min of anoxia (pHo 6.4) and 15 min of reoxygenation (pHo 7.4) in control cells (○, n = 17) and cells treated during anoxia with 20 µmol/l KB (□, n = 16). Data are means ± SE. No differences in cytosolic pH at end of anoxia and during reoxygenation were found between control and KB-treated cells.](image-url)

![Fig. 5. Rise of Mg-fura 2 ratio (original recording, arbitrary units) and change in cell length (% to the initial length) in a cardiomyocyte during anoxia. After 22 min of anoxia the cardiomyocyte developed rigor contracture and rise of the Mg-fura 2 ratio was ended.](image-url)

![Fig. 6. Degree of hypercontracture (reduction of cell length as percentage of normoxic control length) developed during 15 min of reoxygenation in control cells (n = 51) and in cells treated during anoxia with KB at concentrations of 10 µmol/l (n = 44) and 20 µmol/l (n = 29). Data are means ± SE. *P < 0.05 vs. control. Anoxic treatment with KB reduced hypercontracture dose-dependently.](image-url)
In the normoxic heart, the total activities of CK and LDH were 3,604 ± 286 and 2,820 ± 332 U/g dry wt, respectively. During the whole period of anoxia, only a small enzyme release was observed in control hearts (<2% from the total tissue enzyme activity), and KB treatment did not significantly influence this anoxic enzyme release. In contrast, pronounced enzyme release (>15%) was observed in control hearts with the beginning of reoxygenation (Fig. 7). Treatment of the hearts with KB solely during anoxia reduced reoxygenation-induced enzyme release in a dose-dependent manner (Fig. 8).

**DISCUSSION**

**Main Findings and Model Features**

The aim of this study was to investigate the effects of treatment during anoxia with the novel inhibitor of the reverse mode of NCE KB-R7943 on anoxic cation homeostasis and reoxygenation-induced injury. The main findings are the following.

1) Presence of the NCE inhibitor during simulated ischemia significantly reduced cytosolic Ca\(^{2+}\) and Na\(^{+}\) overload in isolated cardiomyocytes but had no effect on energy loss and changes in cytosolic pH.

2) During reoxygenation, cardiomyocytes were protected against reoxygenation-induced hypercontracture when KB had been present during simulated ischemia.

3) Treatment with KB during anoxic perfusion protected the whole heart against reoxygenation-induced enzyme release.

The model of isolated cardiomyocytes exposed to conditions of simulated ischemia and reoxygenation has been characterized in previous studies (11–13). During oxygen depletion, cells develop a deficit of energy that eventually causes a rigor-mediated partial shortening of the myofibrils. H\(^{+}\), Na\(^{+}\), and Ca\(^{2+}\) accumulate in the cytosol. During anoxic conditions of up to 80 min, this ionic imbalance is rapidly reversed on reoxygenation (12). During the initial 5 min of reoxygenation, cells develop hypercontracture, i.e., their length is reduced to approximately one-third of normoxic length. It has been shown that hypercontracture results from the combination of energy resupply due to reoxygenation and cytosolic Ca\(^{2+}\) overload accumulated during anoxia (3, 29). Therefore, the course of anoxia-reoxygenation injury in this model contains two major sequential steps: 1) Ca\(^{2+}\) overload developing during anoxia, and 2) hypercontracture on reoxygenation enabled by the anoxic Ca\(^{2+}\) overload. Reoxygenation-induced hypercontracture is the main underlying cause for severe reoxygenation-induced injury in myocardial tissue (4, 23, 24).

In this study we used this cellular model to characterize the cardioprotective properties of the novel inhibitor of NCE KB-R7943. It was shown by others that this agent inhibits the reverse mode of NCE more potently than the forward mode of NCE or any other major sarcolemmal cation transport mechanism (9, 31). In isolated cardiomyocytes, KB fully inhibits the reverse mode of the NCE at a concentration of 10 µmol/l when the pH\(_{o}\) is 7.4 (9). In this study we analyzed additionally the inhibitory capacity of KB at pH\(_{o}\) 6.4, since this pH was used under conditions of simulated ischemia. It was found that at pH\(_{o}\) 6.4 20 µmol/l, KB is needed for a complete inhibition of the reverse mode of NCE.

**Effects of KB on Cytosolic Ion Homeostasis During Simulated Ischemia**

In anoxic cardiomyocytes, development of rigor contraction, indicating exhaustion of ATP reserves, is followed by the rapid accumulation of Ca\(^{2+}\) in the cytosol (21). In adult cardiomyocytes under ischemic conditions, three ion transport mechanisms may participate in cytosolic Ca\(^{2+}\) overload, namely, the Ca\(^{2+}\) release...
channels of the sarcoplasmic reticulum (28), the sarcolemmal L-type Ca\(^{2+}\) channels (28), and the reverse mode of the sarcolemmal NCE (6, 16, 32). In our model the first two routes play a minor role in cytosolic Ca\(^{2+}\) accumulation, because treatment of cardiomyocytes during anoxia with functionally blocking concentrations of ryanodine or nifedipine had no effect on the development of Ca\(^{2+}\) overload. In contrast, application of 20 µmol/l KB, the concentration that completely inhibited the reverse mode of NCE in normoxic and anoxic cells, reduced the rate of anoxic Ca\(^{2+}\) accumulation by ~80%. This concentration of KB did not affect the time of rigor contracture and, subsequently, the onset of cytosolic Ca\(^{2+}\) overload. A short methodological consideration seems required at this point. We monitored indirectly the changes in the cytosolic Ca\(^{2+}\) concentration by determination of the fura 2 ratio. Fura 2 fluorescence may be influenced by pH\(_i\) (7). This cannot account for the differences in the fura 2 ratio at the end of anoxia, however, because at this point pH\(_i\) was the same under all experimental conditions (Fig. 4). Taken together these results indicate that the reverse mode of NCE accounts for the largest part of Ca\(^{2+}\) overload in the anoxic, energy-depleted cardiomyocytes.

In recent studies, the importance of the reverse mode of NCE in energy-depleted cells has been questioned (8, 15). The doubts are based on the observation that the activity of the NCE may be significantly suppressed under conditions of metabolic inhibition and that dephosphorylation of the NCE may cause its inactivation (8). We also found that the rapid activation of the reverse mode of NCE by Na\(^+\) withdrawal protocol is attenuated after anoxic energy depletion. This indicates indeed an inactivation of the NCE. The fact that KB so effectively suppresses the anoxic development of Ca\(^{2+}\) overload is not in conflict with these observations. It only indicates that the activation of the reverse mode of NCE, even if relatively low, is sufficient to cause accumulation of Ca\(^{2+}\) in the cytosol of energy-depleted cardiomyocytes.

KB has been shown to not be a very selective inhibitor of NCE. It can interfere with other ion transport systems at concentrations in the 10\(^{-5}\) mol/l range (31). Therefore, during anoxia the effect of KB on cytosolic accumulation of H\(^+\) and Na\(^+\), the ions that play an important role in pathogenesis of myocardial ischemia and reperfusion, was tested (22, 30). At 20 µmol/l KB did not have an effect on the cytosolic acidosis at the end of anoxia but significantly reduced the rate of anoxic Na\(^+\) accumulation. This inhibition of Na\(^+\) overload is not consistent with a selective inhibition of the reverse mode of the NCE and must, therefore, have another cause. It cannot be attributed to an inhibition of Na\(^+\)/H\(^+\) exchanger, because the presence of KB during anoxia had no effect on the development of cytosolic acidosis in anoxic cardiomyocytes. We demonstrated in a previous study using the same model (11), that inhibition of the Na\(^+\)/H\(^+\) exchanger causes a pronounced acceleration of cytosolic acidosis if, as done in the present study, HEPES-buffered anoxic medium is used to simulate ischemia. The nature of the route of anoxic Na\(^+\) influx, affected by KB, remains at present unclear.

The side effect of KB on the anoxic Na\(^+\) overload makes it even more attractive as a cardioprotective agent. First, reduction of Na\(^+\) overload may indirectly inhibit the activation of the reverse mode of NCE by modifying the electrochemical gradient and thus potentiating the inhibitory effect of KB. Second, Na\(^+\) overload itself represents an important factor inducing reperfusion arrhythmias (18). Interestingly, in a recent study Nakamura et al. (17) found that treatment with KB dose-dependently reduces the incidence of arrhythmias in the reperfused whole rat hearts.

Taken together, the results obtained with application of KB during simulated ischemia support the indication by previous studies that cytosolic Ca\(^{2+}\) overload is due to activation of the reverse mode of NCE. KB can, by direct and indirect means, interfere with this mechanism.

Protection Against Reoxygenation-Induced Injury

One of the important elements of reperfusion-induced injury of the myocardium is the development of hypercontracture. In isolated cardiomyocytes, where cell-to-cell force transduction is absent, hypercontracture does not induce cell death (21), whereas in tissue it can be a contributing cause for myocardial necrosis (4, 24). Treatment of cardiomyocytes with KB during simulated ischemia reduced dose-dependently the development of hypercontracture during subsequent reoxygenation. This protection cannot be due to an action of KB at the time of reoxygenation, because KB was washed out 10 min before reoxygenation, a time sufficient to remove its effect on activation of the reverse mode of NCE. Previously we found that prolongation of cytosolic acidosis during the reoxygenation phase can prevent reoxygenation-induced hypercontracture (13). However, the protection with KB was not achieved by delayed acidosis. Cytosolic pH before and during reoxygenation was the same in control and KB-treated cells. Protection can be due to a reduction of Ca\(^{2+}\) overload present at the very beginning of reoxygenation. The degree of the end-ischemic Ca\(^{2+}\) overload is an important determinant of reoxygenation-induced hypercontracture (19). In the present study, end-ischemic Ca\(^{2+}\) overload and hypercontracture were both reduced to a similar extent by treatment with 10 or 20 µmol/l KB. This indicates that the protection on reoxygenation is indeed due to the reduction in end-ischemic Ca\(^{2+}\) overload.

To investigate whether the protection against reoxygenation-induced injury observed in isolated cardiomyocytes after anoxia KB treatment is also valid for the whole heart, we exposed isolated Langendorff-perfused rat hearts to anoxia and reoxygenation. After a sufficient time of oxygen depletion, pronounced reoxygenation-induced injury, known as "oxygen paradox," can be observed (24). In the present study hearts were exposed to anoxic perfusion with medium pH 6.4, because this medium pH was also applied to the isolated cardiomyocytes. It was found that the presence
of KB during anoxic perfusion reduced dose-dependently the reoxygenation-induced enzyme release. Taken together, the results demonstrate that KB protects myocardial cells against severe injury induced during the early phase of reoxygenation. A recent study by Nakamura et al. (17) has shown that pretreatment with KB before global ischemia can protect against postischemic contractile dysfunction. It is unclear whether this protective effect on cardiac function has the same cause as the one here described.

In conclusion, this study shows that treatment during anoxia with KB at a concentration that completely inhibits the reverse mode of Na+/Ca2+ exchanger, significantly reduces cytosolic Ca2+ and Na+ overload. It has no effect on cytosolic acidosis and energy depletion. Treatment during anoxia with KB offers benefits not only during oxygen depletion but also during reoxygenation. Reoxygenation-induced hypercontracture of isolated cardiomyocytes as well as reoxygenation-induced enzyme release of whole hearts were found to be greatly attenuated. These findings indicate that inhibitors of the reverse mode of NCE, like KB, represent valuable means to protect ischemic-reperfused myocardium against injury developing both during ischemia and on reperfusion.

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