Protection of Rat Cardiomyocytes Against Simulated Ischemia and Reoxygenation by Treatment With Protein Kinase C Activator

Y.V. Ladilov, C. Balser, H.M. Piper

Abstract—The aim of this study was to investigate whether treatment with the protein kinase C (PKC) agonist 1,2-dioctanoyl-sn-glycerol (1,2DOG) can protect isolated adult Wistar rat cardiomyocytes against simulated ischemia and reoxygenation. Cytosolic Ca\(^{2+}\) (assessed by fura 2 fluorescence), pH\(_i\) (assessed by BCECF fluorescence), and cell length were measured during 80 minutes of simulated ischemia (anoxia, pH\(_o\) 6.4) and 20 minutes of reoxygenation (pH\(_o\) 7.4) and compared between control cells and cells treated with 20 \(\mu\)mol/L 1,2DOG before anoxia (10-minute treatment and 10-minute washout), before and during anoxia (two-step treatment), or only during anoxia. Treatment before anoxia attenuated rigor contracture but did not influence anoxic Ca\(^{2+}\) overload. In contrast, two-step treatment before and during anoxia accelerated rigor contracture but reduced the rate of anoxic Ca\(^{2+}\) accumulation. During reoxygenation, control cells developed irreversible hypercontracture (reduction of cell length to 43±2% of the initial cell length, \(n=62\)), which was accompanied by spontaneous oscillations of cytosolic Ca\(^{2+}\) (19.6±1.6 per minute). Two-step treatment with 1,2DOG before and during anoxia significantly reduced hypercontracture (reduction of cell length to 60±2%, \(P<.01\) versus control, \(n=41\)) and suppressed spontaneous Ca\(^{2+}\) oscillations (2.8±0.9 per minute, \(P<.01\) versus control). These effects could not be reproduced by treatment with 1,2DOG before anoxia or during anoxia or by a two-step treatment with the PKC-inactive 1,3-dioctanoyl-sn-glycerol and were fully abolished with 1 \(\mu\)mol/L bisindolylmaleimide (PKC inhibitor). We conclude that a two-step activation of PKC before and during anoxia is required for effective protection of cardiomyocytes against anoxic Ca\(^{2+}\) overload and reoxygenation-induced hypercontracture. (Circ Res. 1998;82:451-457.)

Key Words: protein kinase C  ■  dioctanoyl-sn-glycerol  ■  cardiomyocyte  ■  ischemia  ■  reoxygenation

During ischemia, cardiomyocytes develop cytosolic Ca\(^{2+}\) overload. This Ca\(^{2+}\) overload is a determinant not only of cellular injury developing during ischemia but also of reoxygenation-induced injury. One of the important elements of this injury is hypercontracture, caused by the resupply of energy to myofibrils at cytosolic Ca\(^{2+}\) overload.1,2 Recently, it has been shown that myocardial injury induced by ischemia/reperfusion is markedly reduced by ischemic PC, i.e., when a prolonged exposure to ischemia is preceded by one or more brief ischemic episodes.3 Activation of PKC has been shown to be an important element in the cardioprotective mechanism of ischemic PC.4,5 Apart from its involvement in protection by ischemic PC, little is known about the effects of PKC activation on the course of ischemia/reperfusion injury in myocardium. The investigation of this question in whole heart is complicated, because the intact heart is a complex of tissues and because direct effects of PKC stimulation on cardiomyocytes can be masked by side effects on other cells (e.g., mast cells, endothelial cells, and neurons), as well as by hemodynamic effects.

In the present study, we addressed the question whether direct stimulation of PKC can protect cardiomyocytes against injury induced by simulated ischemia (Ca\(^{2+}\) overload) and reperfusion (hypercontracture). We used the model of simulated ischemia/reperfusion in isolated rat cardiomyocytes, which was characterized in detail in previous studies.6,7 For stimulation of PKC, 1,2DOG, a membrane-permeable diacylglycerol analogue, was used. To separate PKC-dependent effects of 1,2DOG treatment from side effects, cells were treated with PKC-inactive 1,3DOG. Three different protocols were performed: with the first, cells were treated with 20 \(\mu\)mol/L 1,2DOG only before anoxia; with the second, cells were treated with 1,2DOG before and during anoxia; and with the third, cells were treated with 1,2DOG only during anoxia.

Materials and Methods

Preparation of Isolated Cardiomyocytes

Ventricular heart muscle cells were isolated from 200- to 250-g adult male Wistar rats and plated in medium 199 with 4% fetal calf serum on glass coverslips that had been preincubated overnight with 4% fetal calf serum.2 Four hours after plating, 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. Three different protocols were performed: with the first, cells were treated with 20 \(\mu\)mol/L 1,2DOG only before anoxia; with the second, cells were treated with 1,2DOG before and during anoxia; and with the third, cells were treated with 1,2DOG only during anoxia.

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From the Physiologisches Institut, Justus-Liebig-Universität, Giessen, Germany.
Correspondence to Prof H.M. Piper, Physiologisches Institut, Justus-Liebig-Universität, Aulweg 129, D-35392 Gießen, Germany.
E-mail michael.piper@physiologie.med.uni-giessen.de
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cardiomyocytes attached to the coverslip. From each isolation two or three coverslips were used. On each coverslip, from four to six cells were investigated. Only cells exhibiting a rod-shaped morphology and no signs of sarcosomal blebbing were used for the experiments. These cells were found to have a low resting [Ca\(^{2+}\)], (see below).

### Ca\(^{2+}\), pH, and Cell Length Measurements

To measure [Ca\(^{2+}\)], or [H\(^+\)], cardiomyocytes were loaded in medium 199 at 35°C for 30 minutes with acetoxyethyl esters of fura 2 (2.5 \(\mu\)mol/L) or BCECF (1.5 \(\mu\)mol/L), respectively. After the loading, cells were washed twice with medium 199. This was followed by incubation in medium 199 for 30 minutes to allow hydrolysis of the acetoxyethyl esters within the cell. The fluorescence from dye-loaded cells was 20 to 30 times higher than background fluorescence from unloaded cells.

The coverslip with loaded cells was introduced into a gastight, temperature-controlled (37°C), transparent perfusion chamber positioned in the light path of an inverted microscope (Diaphot TMD, Nikon). Alternating excitation of the fluorescent dye at wavelengths of 340 and 380 nm for fura 2 or 450 and 490 nm for BCECF was performed with an AR-Cation Measurement System adapted to the microscope (Spex Industries). Emitted light (490 to 510 nm for fura 2 and 520 to 560 nm for BCECF) from a 10×10-mm 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 DM30000CM, Spex Industries).

In the analysis of the fura 2 signal in reoxygenated cardiomyocytes, the following parameters were determined: (1) “diastolic” fura 2 ratio, i.e., the fura 2 ratio when not oscillating or curve of minima when oscillating, and (2) frequency of oscillations of the fura 2 ratio at given times.

Simultaneous to the measurement of the fluorescence, the cell microscropic image was recorded with a video camera and stored on tape. From these recordings, changes of the cell length were determined later. In the case of hypercontracted cells, the cell dimension along its previous longitudinal axis was determined.

### Dye 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 test the extent of intracellular dye compartmentation cells were chemically “skinned” with digitonin as described previously. Briefly, cardiomyocytes were metabolically inhibited with 1 mmol/L KClN to prevent hypercontracture during calibration and superfused for 5 minutes with EGTA buffer containing (mmol/L) KCl 135, NaCl 5, HEPES 5, EGTA 1, and KCN 1, pH 7.2 at 37°C. After this procedure, 2.5 \(\mu\)mol/L digitonin was added. Digitonin permeabilizes the sarcosomal membrane but leaves membranes of organelles intact. After release of the dyes from the cytosol, the residual fluorescence was measured, which was a sum of fluorescence from compartment and background fluorescence. To separate them, 1 mmol/L MnCl\(_2\) and 5 mmol/L ionomycin were added to the buffer. This quenched the fluorescence of the dyes within organelles, leaving background fluorescence. The background fluorescence was subtracted from the initial fluorescence. Excitation of the fluorescent dyes was performed at wavelengths of 360 nm for fura 2 and 450 nm for BCECF. This test showed that the fluorescent signal from intracellular stores did not exceed 10% for fura 2 and 12% for BCECF compared with the signal from whole cells. Furthermore, the 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 and BCECF

Because of the inherent problems with calibration of the fura 2 ratio, data were generally expressed in arbitrary units of fluorescence ratio. Control (before anoxia) and end-anoxic values of the fura 2 signal were calibrated according to the method described by Li et al with modifications. The cells were exposed to 5 mmol/L ionomycin and 10 mg/ml nigericin in solution (pH 6.35 and 7.15) containing (mmol/L) NaCl 10, KCl 125, MgSO\(_4\) 1, and HEPES 25, along with either 3 mmol/L Ca\(^{2+}\) or 5 mmol/L EGTA to obtain, respectively, the maximum (R\(_{\text{max}}\)) and the minimum (R\(_{\text{min}}\)) ratio of fluorescence. To prevent morphological alterations during calibration, cells were ATP-depleted with 1 mmol/L KCN. [Ca\(^{2+}\)], was calculated according to the following equation: [Ca\(^{2+}\)] = \(K_d \times R - R_{\text{max}} \)/(R_{\text{max}} - R), where \(K_d\) is the ratio of the 380-nm excitation signals of ionomycin-treated cells at 3 mmol/L EGTA and at 3 mmol/L Ca\(^{2+}\), and \(K_d\) is the dissociation constant of fura 2. For fura 2 dissolved in buffer, Grynkiewicz et al determined a \(K_d\) of 224 mmol/L (K\(_d\) in vitro). However, the affinity of fura 2 to Ca\(^{2+}\) inside a cell may differ from the affinity in solution. We found that at pH 7.15 the \(K_d\) in intact cells was higher than the \(K_d\) in solution (312±9 mmol/L [n = 8] versus 200±11 mmol/L [n = 7]). At pH 6.5, the intracellular \(K_d\) was found to be 337±4 mmol/L (n = 6). The conversion of fura 2 ratios into absolute values of intracellular Ca\(^{2+}\) was performed using intracellular \(K_d\) values. Calibration of the BCECF ratio signal was performed, as previously described by Koop and Piper, with 10 mg/ml nigericin, a K\(^+-\)H\(^+\) ionophore, and incubation media with various pH values.

### Media

The perfusion chamber (0.5-mL filling volume) was perfused at a flow rate of 0.6 mL/min with modified glucose-free Tyrode’s solution containing (mmol/L) Na\(_2\)Cl 135.0, KCl 2.6, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 1.2, CaCl\(_2\) 1.0, and HEPES 25.0; pH was 7.4 at 37°C. Medium was made anoxic by autoclaving as described previously and was equilibrated before and during use with 100% N\(_2\). Normoxic medium was equilibrated with air.

### Experimental Protocols

The standard anoxia/reoxygenation protocol included 80 minutes of anoxia at 37°C and pH 6.4 and 20 minutes of reoxygenation in medium at pH 7.4. This protocol has been shown previously to produce rigor contracture, cytosolic Ca\(^{2+}\) overload (pCa 5/6), and acidosis (pH, 6.5) during anoxia and irreversible hypercontracture during reoxygenation that results in depletion of the cellular state of energy and tonic homeostasis on reoxygenation. The time of rigor contracture is variable in anoxic cardiomyocytes. Once it has occurred, Ca\(^{2+}\) rises in a predictable manner. Under control conditions it takes, at most, 30 minutes after rigor contracture to develop anoxic Ca\(^{2+}\) overload. Since the total period of anoxia was continued for 80 minutes, only those cells were included in the study in which rigor contracture occurred within the first 50 minutes.

Seven sets of experiments were performed. The first group of experiments was performed (Fig 1). In all of them, cells were first exposed to 20 minutes of normoxia at pH, 7.4, followed by 80 minutes of anoxia at pH, 6.4 (simulated ischemia) and 20 minutes of reoxygenation at pH, 7.4 (simulated reperfusion). In the control experiments (protocol 1), this standard protocol was performed without modification. In protocol 2 (+1,2DOG), the first normoxic superfusion of cells was started with 1,2DOG (20 \(\mu\)mol/L) present for the first 10 minutes. It was washed out for the subsequent 10 minutes. In protocol 3 (+1,2DOG+), cardiomyocytes were treated with 1,2DOG before and during anoxia. In protocol 4 (1,2DOG+), 1,2DOG was present only during anoxia. In protocol 5, the PKC-inactive 1,3DOG (20 \(\mu\)mol/L) was administered before and during anoxia, analogously to protocol 3. In protocol 6, the control conditions of protocol 1 were applied with the addition of the continuous presence of the PKC inhibitor with 1 \(\mu\)mol/L BIM.
during 20 minutes normoxia and subsequent 80 minutes anoxia. Protocol 7 was similar to protocol 3 but 1 μmol/L BIM was continuously present until just before reoxygenation.

Materials
Medium 199 was purchased from Boehringer-Mannheim; fetal calf serum, from GIBCO; acetoxyethyl esters of fura 2 and BCECF, from Paesel and Lorey; 1,2DOG and 1,3DOG, from Sigma Chemical Co; and BIM, from Calbiochem-Novabiochem. All other chemicals were from Merck and were of the highest purity available.

Statistics
Data are given as mean±SE. For each experimental protocol, 20 to 60 individual cells were used, with not more than six cells from the same cell isolates. Statistical comparisons were performed by one-way ANOVA and use of the Bonferroni test for post hoc analysis.

Results
Influence of Treatment With 1,2DOG on Cytosolic Ca2+ Overload During Anoxia
Normoxic cardiomyocytes were quiescent and had [Ca2+]i of 44±5 nmol/L and pH 7.18±0.05. In the control group (protocol 1), superfusion of cardiomyocytes with anoxic glucose-free medium with pH 6.4 caused accumulation of Ca2+ in the cytosol, as indicated by an increase in the fura 2 ratio (Fig 2, control). [Ca2+]i at the end of anoxia was 1.96±0.07 μmol/L (n = 62). The accumulation of Ca2+ started 30 minutes after the beginning of anoxia, directly after the cells developed rigor contracture. When cells were reoxygenated after 80 minutes of anoxia, the fura 2 ratio recovered to its initial level within the next 20 minutes.

In protocol 2, 20 μmol/L 1,2DOG was applied for 10 minutes to normoxic cells and then washed out for another 10 minutes. When the cells were exposed to anoxia after this pretreatment, the fura 2 ratio rose with a rate similar to that of the control group. At the end of anoxia, [Ca2+]i, reached 2.16±0.10 μmol/L (n = 40) (Fig 2, +1,2DOG). In protocol 3, the additional treatment with 1,2DOG during anoxia had a pronounced effect on the development of anoxic Ca2+ overload. In this group, the fura 2 ratio started to rise earlier but was significantly slower than in protocols 1 and 2 (Fig 2, +1,2DOG+). At the end of anoxia in protocol 3, the extent of Ca2+ overload was significantly lower than in protocols 1 and 2 (0.61±0.05 μmol/L, n = 41, P<.01 versus protocol 1). When 1,2DOG was applied only during anoxia (protocol 4), the developing anoxic Ca2+ overload was only moderately reduced compared with protocol 3 (1.35±0.10 μmol/L, n = 31, P<.01 versus protocols 1 and 3) (Fig 2, 1,2DOG+). In protocol 5, cells were treated with PKC-inactive 1,3DOG before and during anoxia. No significant effects on anoxic Ca2+ overload (1.81±0.11, n = 22) were found compared with the control situation (Fig 3, 1,3DOG+).

Influence of Treatment With 1,2DOG on the Time of Rigor Contracture
As shown before,7 cardiomyocytes eventually shorten when their energy stores are depleted. This shortening is due to a rigor mechanism. It is a rapid process of cell length reduction by about one third within 30 seconds. Under control condi-

Figure 1. Experimental protocols. The numbers under the bars represent the duration of each period. Black bar indicates treatment with 20 μmol/L dioctanoyl-sn-glycerol; O2 pH 7.4, aerobic superfusion, extracellular pH 7.4; N2 pH 6.4, anaerobic superfusion, extracellular pH 6.4; 1,2DOG, treatment with 20 μmol/L PKC-active 1,2DOG; 1,3DOG, treatment with 20 μmol/L PKC-inactive 1,3DOG; and BIM, treatment with 1 μmol/L BIM before (20 minutes) and during (80 minutes) anoxia. Black bar indicates treatment.

Figure 2. Time courses of fura 2 ratio (arbitrary units) in single cardiomyocytes during anoxia and reoxygenation. The following experimental protocols are shown: control (○, n = 62), +1,2DOG (●, n = 40), +1,2DOG+ (●, n = 41), 1,2DOG+ (●, n = 31), and +1,3DOG+ (●, n = 22). Protocols are explained in Fig. 1 Data are mean±SE. *P<.01 vs control.

Figure 3. Time until onset of rigor contracture in single cardiomyocytes during superfusion with anoxic medium. The following experimental protocols are shown: control (○, n = 62), +1,2DOG (●, n = 40), +1,2DOG+ (●, n = 41), 1,2DOG+ (●, n = 31), and +1,3DOG+ (●, n = 22). Protocols are explained in Fig. 1 Data are mean±SE. *P<.01 vs control.
Hypercontracture by 1,2DOG Treatment

Marked protection against reoxygenation-induced hypercontraction was found when cells were treated before anoxia (protocol 2) slightly reduced hypercontracture. Application of 1,2DOG only during anoxia or treatment with 1,3DOG had no significant effect on the development of hypercontracture, however, was found when cells were treated before and during anoxia with 1,2DOG (protocol 3). Treatment with 1,2DOG only during anoxia (protocol 4) or with 1,3DOG (protocol 5) had no effect on the onset of rigor shortening.

In an additional set of experiments, the Na\(^+\)-H\(^+\) exchanger was blocked by the addition of HOE 642 (3 \(\mu\)mol/L). Inhibition of the exchanger significantly enhanced development of acidosis during anoxia. The extent of acidosis was the same in cells under control conditions and protocol 3, indicating that treatment with 1,2DOG did not affect anoxic H\(^+\) production.

Pharmacological Preconditioning

Protection of Cardiomyocytes Against Hypercontracture by 1,2DOG Treatment

During anoxia the cell length was reduced by rigor shortening to a similar degree under all experimental conditions. Reoxygenation of cells in the control group led to irreversible extreme reduction of cell length, ie, hypercontracture. In the control group, the frequency of the oscillations was 19.6±1.6 per minute (n=62) (Fig 4). Pretreatment with 1,2DOG (protocol 2) had no effect on the oscillations. Application of 1,2DOG before and during anoxia (protocol 3) significantly reduced these oscillations to 2.8±0.9 minute per minute (n=61). Application of 1,2DOG only during anoxia or treatment with 1,3DOG had no effect on the oscillation frequency of the fura 2 ratio. In spite of these differences among oscillations, the “diastolic” fura 2 ratio, ie, the fura 2 ratio when not oscillating or the minima of the fura 2 ratio when oscillating, declined with similar rates under all experimental conditions and reached the initial control level within 20 minutes (Fig 2). This indicated that overall Ca\(^{2+}\) control is recovered with comparable rapidity in all protocols.

Influence of Treatment With 1,2DOG on Recovery of Cytosolic Ca\(^{2+}\) During Reoxygenation

The recovery of the fura 2 ratio during reoxygenation passed through a short period of spontaneous oscillations. In the control group, the frequency of the oscillations was 19.6±1.6 per minute (n=62) (Fig 4). Pretreatment with 1,2DOG (protocol 2) had no effect on the oscillations. Application of 1,2DOG before and during anoxia (protocol 3) significantly reduced these oscillations to 2.8±0.9 per minute (n=61). Application of 1,2DOG only during anoxia or treatment with 1,3DOG had no effect on the oscillation frequency of the fura 2 ratio. In spite of these differences among oscillations, the “diastolic” fura 2 ratio, ie, the fura 2 ratio when not oscillating or the minima of the fura 2 ratio when oscillating, declined with similar rates under all experimental conditions and reached the initial control level within 20 minutes (Fig 2). This indicated that overall Ca\(^{2+}\) control is recovered with comparable rapidity in all protocols.

Inhibition of PKC With BIM Abolishes Beneficial Effects of 1,2DOG Treatment

To strengthen the argument that beneficial effects of two-step treatment with 1,2DOG are indeed PKC dependent, PKC was inhibited by treatment with 1 \(\mu\)mol/L BIM before anoxia (20

### Table 1. pH\(_i\) in Normoxic, Anoxic, and Reoxygenated Cardiomyocytes

<table>
<thead>
<tr>
<th>Protocols</th>
<th>Normoxia</th>
<th>Anoxia 80 Min</th>
<th>Anoxia 5 Min</th>
<th>Anoxia 20 Min</th>
<th>Reoxygenation 5 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=29)</td>
<td>7.12±0.02</td>
<td>6.57±0.01</td>
<td>6.73±0.02</td>
<td>7.07±0.04</td>
<td></td>
</tr>
<tr>
<td>+1,2DOG (n=26)</td>
<td>7.14±0.02</td>
<td>6.58±0.03</td>
<td>6.76±0.03</td>
<td>7.12±0.02</td>
<td></td>
</tr>
<tr>
<td>+1,2DOG+ (n=31)</td>
<td>7.10±0.01</td>
<td>6.56±0.02</td>
<td>6.77±0.03</td>
<td>7.15±0.04</td>
<td></td>
</tr>
<tr>
<td>1,2DOG (n=26)</td>
<td>7.09±0.01</td>
<td>6.54±0.03</td>
<td>6.77±0.04</td>
<td>7.14±0.02</td>
<td></td>
</tr>
<tr>
<td>Control plus HOE 642 (n=24)</td>
<td>7.18±0.03</td>
<td>5.67±0.05</td>
<td>5.97±0.08</td>
<td>7.12±0.02</td>
<td></td>
</tr>
<tr>
<td>+1,2DOG+ plus HOE 642 (n=25)</td>
<td>7.15±0.02</td>
<td>5.71±0.07</td>
<td>5.91±0.08</td>
<td>7.06±0.06</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SE. Protocols are explained in Fig 1. No significant differences were observed between control cells and cells treated with 1,2DOG.

### Changes of pH\(_i\) During Anoxia and Reoxygenation

As described previously,\(^6,7\) pH\(_i\) declined in cardiomyocytes exposed to anoxic media with pH 6.4. In the control group (protocol 1), it reached pH 6.57±0.01 (n=29) after 80 minutes of anoxia (Table 1). When protocols 2, 3, and 4 were applied to the cells, intracellular acidosis developed to the same extent. Reoxygenation during 20 minutes in medium with pH 7.4 led to the similar recovery of pH\(_i\) to the initial level under all experimental conditions.

In an additional set of experiments, the Na\(^+\)-H\(^+\) exchanger was blocked by the addition of HOE 642 (3 \(\mu\)mol/L). Inhibition of the exchanger significantly enhanced development of acidosis during anoxia. The extent of acidosis was the same in cells under control conditions and protocol 3, indicating that treatment with 1,2DOG did not affect anoxic H\(^+\) production.

### Figure 4. Oscillation frequency of fura 2 ratio in single cardiomyocytes at the fifth minute of reoxygenation.

The following experimental protocols are shown: control (n=62), +1,2DOG (n=40), +1,2DOG+ (n=41), 1,2DOG+ (n=31), and +1,3DOG+ (n=22). Protocols are explained in Fig 1. Data are mean ±SE. \(^*P<.01\) vs control.
ischemic conditions and subsequent reoxygenation has been able to provide such protection.

only before or only at the onset of simulated ischemia was not depletion and prevented the development of hypercontracture ischemia markedly reduced Ca$^{2+}$ cardiomyocytes with 1,2DOG before and during simulated

Main Findings and Model Features

The aim of the present study was to determine whether activation of PKC can

Effect of 1,2DOG Treatment on Cytosolic Ca$^{2+}$ Overload During Simulated Ischemia

During anoxia, ATP depletion indicated by development of rigor contracture is followed by development of pronounced Ca$^{2+}$ overload. Treatment before anoxia with the PKC activator 1,2DOG significantly delayed the onset of rigor contraction but did not influence the rate of Ca$^{2+}$ overload. In protocol 3, this pretreatment was combined with treatment during anoxia. Under this condition, cardiomyocytes developed rigor contracture earlier, but the rate of Ca$^{2+}$ accumulation was greatly reduced, and at the end of 80 minutes of anoxia, Ca$^{2+}$ overload was significantly lower than in the control cells. A short methodological consideration seems required at this point. We indirectly monitored the changes in cytosolic Ca$^{2+}$ concentration by determination of the fura 2 ratio. Fura 2 fluorescence may be influenced by differences in pH.$^{25}$ This does not account for the differences in the fura 2 ratio at the end of anoxic experiments, however, since at this point pH was the same under all experimental conditions (Table 1). For calculation of the corresponding values of [Ca$^{2+}$], calibration procedures were performed for the pH under control conditions and the pH after 80 minutes of simulated ischemia.

**TABLE 2. Prevention of Protective Effects of Two-Step Treatment With 1,2DOG by Inhibition of PKC With 1 $\mu$mol/L BIM**

<table>
<thead>
<tr>
<th>Protocols</th>
<th>Fura 2 Ratio, AU</th>
<th>Oscillations, min$^{-1}$</th>
<th>Cell Length, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=42)</td>
<td>2.58±0.06</td>
<td>34±3</td>
<td>36±2</td>
</tr>
<tr>
<td>Control+BIM (n=17)</td>
<td>2.61±0.11</td>
<td>36±3</td>
<td>32±2</td>
</tr>
<tr>
<td>1,2DOG+BIM (n=18)</td>
<td>2.49±0.06</td>
<td>31±5</td>
<td>37±3</td>
</tr>
</tbody>
</table>

The fura 2 ratio (after 80 minutes of anoxia) is given in arbitrary units (AU). Oscillation values were after 5 minutes of reoxygenation. Cell length (after 20 minutes of reoxygenation) is given as a percentage of normoxic length. No significant differences were observed between control cells and cells treated with BIM.
The attenuation of \(\text{Ca}^{2+}\) accumulation after the two-step treatment with 1,2DOG (protocol 3) is not due to an unspecified chemical action of 1,2DOG, since the analogous compound, 1,3DOG, which lacks the ability to activate PKC, could not imitate the actions of the PKC activator, 1,2DOG. Furthermore, application of the PKC inhibitor, BIM, eliminated the protection of two-step treatment with 1,2DOG against \(\text{Ca}^{2+}\) overload. The effects of two-step treatment with 1,2DOG could not be reproduced by treatment only during anoxia with 1,2DOG (protocol 4), although under this protocol \(\text{Ca}^{2+}\) overload was slightly attenuated at the end of anoxia.

It was found previously\(^7\) that the cause of \(\text{Ca}^{2+}\) accumulation in this model is an influx of \(\text{Ca}^{2+}\) across the sarcolemma and not a \(\text{Ca}^{2+}\) release from endogenous stores. It was beyond the scope of the present study to analyze further the ways of \(\text{Ca}^{2+}\) influx during anoxia. It was suggested by others that \(\text{Ca}^{2+}\) influx in the anoxic cardiomyocytes is linked indirectly to \(\text{H}^+\) production, since \(\text{H}^+\) extrusion through the \(\text{Na}^-\text{H}^+\) exchanger may lead to \(\text{Na}^+\) accumulation and a secondary \(\text{Ca}^{2+}\) influx through an activation of the \(\text{Na}^-\text{Ca}^{2+}\) exchanger in the “reverse mode.”\(^2\) In the present study, we did not find any difference in intracellular acidosis after treatment with 1,2DOG in the absence or presence of the inhibitor of the \(\text{Na}^-\text{H}^+\) exchanger, HOE 642 (Table 1). Thus, differences in proton production and extrusion through the \(\text{Na}^-\text{H}^+\) exchanger are not likely the causes of the differences in \(\text{Ca}^{2+}\) accumulation.

**Effect of 1,2DOG Treatment on Recovery of \(\text{Ca}^{2+}\) Balance and Hypercontracture During Reoxygenation**

Reoxygenation of cardiomyocytes led to rapid recovery of cytosolic \(\text{Ca}^{2+}\) in all experimental groups. During the initial period of recovery, a burst of spontaneous \(\text{Ca}^{2+}\) oscillations was observed. Treatment of cardiomyocytes with 1,2DOG only before or only during anoxia had no effect on the frequency of these oscillations. A pronounced inhibition of the oscillations was observed, however, after two-step treatment with 1,2DOG before and during anoxia. Previous data\(^2\) have shown that these \(\text{Ca}^{2+}\) oscillations are due to cyclic shifts of \(\text{Ca}^{2+}\) between the cytosol and the SR and occur when the extent of \(\text{Ca}^{2+}\) overload at the beginning of reoxygenation exceeds the capacity of the SR. They last until the major part of \(\text{Ca}^{2+}\) overload is extruded from the cytosol. The oscillations reached their maximal frequency at the 5th minute of reoxygenation and expired by the 10th minute of reoxygenation.\(^5\) Suppression of the oscillations observed under protocol 3 was not due to a prolongation of intracellular acidosis (Table 1). It is likely that the reduction in oscillatory \(\text{Ca}^{2+}\) movement is due to the reduction in the extent of \(\text{Ca}^{2+}\) overload at the end of anoxia.

Hypercontracture is an important element of reoxygenation-induced injury in isolated cardiomyocytes. In this cell model, hypercontracture does not induce cell death,\(^3,\)\(^4\) whereas in tissue it can be a contributing cause for myocardial necrosis.\(^25\) After two-step treatment with 1,2DOG before and during anoxia (protocol 3), hypercontracture was almost completely suppressed. Treatment with 1,2DOG only before or only during anoxia could not reproduce this beneficial effect. Previously, we have shown that prolongation of cytosolic acidosis during the reoxygenation phase can prevent reoxygenation-induced hypercontracture.\(^3\) In the present study, though, the protection was not achieved by delayed acidosis, since pH before and during reoxygenation was the same in control and 1,2DOG-treated cells. The protection seems rather to be due to reduction of cytosolic \(\text{Ca}^{2+}\) overload at the beginning of reoxygenation and the related reduction in \(\text{Ca}^{2+}\) oscillations, which represent temporal \(\text{Ca}^{2+}\) overload. Since after two-step treatment with 1,2DOG these spontaneous oscillations of cytosolic \(\text{Ca}^{2+}\) were significantly depressed, the protective effect may be, at least partly, attributed to depression of the oscillations. None of the beneficial effects of two-step treatment with 1,2DOG was observed when the PKC inhibitor BIM was present until just before reoxygenation. This observation also supports the conclusion that treatment with 1,2DOG acts through PKC activation.

**Relation to Ischemic Preconditioning**

In intact myocardium, the protective effect of ischemic PC may be caused by the interstitial accumulation of endogenous mediators like adenosine and noradrenaline during PC and sustained ischemia, which elicit activation of PKC via stimulation of sarcolemmal receptors. In the present study, we applied an artificial stimulation of PKC with 1,2DOG. This approach was chosen, because in our model sufficient accumulation of these mediators during simulated ischemia and therefore receptor-mediated PKC activation seems not feasible.

Nevertheless, the protection of anoxic/reoxygenated cardiomyocytes by PKC activation in the present study resembles in a number of aspects the protective mechanism of ischemic PC in ischemic/reperfused myocardium.

The protective effect of PKC stimulation was observed only if it occurred before and during simulated ischemia. Similarly, ischemic PC of whole hearts has been shown to depend on PKC activation before and at the onset of prolonged ischemia.\(^4,\)\(^28\) Armstrong and Ganote\(^27\) found that in isolated cardiomyocytes, activity of PKC during sustained simulated ischemia is required when the cells are to be protected by PC protocol.

In the present study, the observed protective effects of the two-step treatment with 1,2DOG, ie, reduction of anoxic \(\text{Ca}^{2+}\) overload, paradoxical acceleration of ischemic rigor contracture, and suppression of SR-dependent cytosolic \(\text{Ca}^{2+}\) shifts during reoxygenation, were also observed in isolated hearts on ischemic PC.\(^28\)\(^30\) These similarities suggest that in the present study, the investigated model of anoxic/reoxygenated cardiomyocytes under PKC stimulation represents, at least partly, a cellular model of the protective mechanism of ischemic PC. The finding that PKC activation can protect against reoxygenation-induced hypercontracture of the cardiomyocytes represents an effect that has not yet been identified for ischemic PC. It would certainly be worth investigating whether the protective effect of ischemic PC could, in part, be attributed to protection against reoxygenation-induced hypercontracture.
Conclusion
The results of the present study show that two-step treatment with a PKC activator before and during simulated ischemia protects isolated cardiomyocytes, both against Ca\(^{2+}\) overload developing under the ischemic conditions and reoxygenation-induced hypercontracture. The effects on the reoxygenation phase are (1) attenuation of cytosolic Ca\(^{2+}\) oscillations, which may lead to reperfusion arrhythmias in the ischemic/reperfused heart in vivo, and (2) reduction of hypercontracture, which may cause contraction-band necrosis in vivo.

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References
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Y. V. Ladilov, C. Balser and H. M. Piper

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