mitoK\textsubscript{ATP} channel activation in the postanoxic developing heart protects E-C coupling via NO-, ROS-, and PKC-dependent pathways

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Sarre, Alexandre, Norbert Lange, Pavel Kucera, and Eric Raddatz. mitoK\textsubscript{ATP} channel activation in the postanoxic developing heart protects E-C coupling via NO-, ROS-, and PKC-dependent pathways. Am J Physiol Heart Circ Physiol 288: H1611–H1619, 2005. First published November 18, 2004; doi:10.1152/ajpheart.00942.2004.—Whereas previous studies have shown that opening of the mitochondrial ATP-sensitive K\textsuperscript{+} (mitoK\textsubscript{ATP}) channel protects the adult heart against ischemia-reperfusion injury, it remains to be established whether this mechanism also operates in the developing heart. Isolated spontaneously beating hearts from 4-day-old chick embryos were subjected to 30 min of anoxia followed by 60 min of reoxygenation. The chrono-, dromo-, and inotropic disturbances, as well as alterations of the electromechanical delay (EMD), reflecting excitation-contraction (E-C) coupling, were investigated. Production of reactive oxygen species (ROS) in the ventricle was determined using the intracellular fluorescent probe 2',7'-dichlorofluorescin (DCFH). Effects of the specific mitoK\textsubscript{ATP} channel opener diazoxide (Diazo, 50 \textmu M) or the blocker 5-hydroxydecanoate (5-HD, 500 \textmu M), the nitric oxide synthase (NOS) inhibitor N\textsuperscript{G}-nitro-l-arginine methyl ester (l-NAME, 50 \textmu M), the antioxidant N-(2-mercaptopropionyl)glycine (MPG, 1 \textmu M), and the PKC inhibitor chelerythrine (Chel, 5 \textmu M) on oxidative stress and postanoxic functional recovery were determined. Under normoxia, the baseline parameters were not altered by any of these pharmacological agents, alone or in combination. During the first 20 min of postanoxic reoxygenation, Diazo doubled the peak of ROS production and, interestingly, accelerated recovery of ventricular EMD and the PR interval. Diazo-induced ROS production was suppressed by 5-HD, MPG, or l-NAME, but not by Chel. Protection of ventricular EMD by Diazo was abolished by 5-HD, MPG, l-NAME, or Chel, whereas protection of the PR interval was abolished by l-NAME exclusively. Thus pharmacological opening of the mitoK\textsubscript{ATP} channel selectively improves postanoxic recovery of cell-to-cell communication and ventricular E-C coupling. Although the NO-, ROS-, and PKC-dependent pathways also seem to be involved in this cardioprotection, their interrelation in the developing heart can differ markedly from that in the adult myocardium.

Diazoxide; mitochondria; chick; hypoxia-reoxygenation; atrioventricular coupling

IT IS GENERALLY ACCEPTED that activation of the mitochondrial ATP-sensitive K\textsuperscript{+} (mitoK\textsubscript{ATP}) channels can protect (8, 26) the heart against ischemia-reperfusion injury (1) and mimic ischemic preconditioning (22). Indeed, mitoK\textsubscript{ATP} channel opening improves the recovery of contractility (7, 40) and reduces infarct size (10, 27) in the reperfused heart. In adult (3, 7, 25, 31), neonatal (14), or embryonic (19, 47) isolated cardiomyocytes, pretreatment with a mitoK\textsubscript{ATP} channel opener appears to increase reactive oxygen species (ROS) production before ischemia and reduce postischemic injury. Furthermore, the protection afforded by mitoK\textsubscript{ATP} channel openers against the reperfusion-induced cell death (apoptotic or necrotic) appears to necessitate stimulation of PKC activity by ROS (24) and activation of nitric oxide (NO)-dependent pathways (19, 33). Despite recent advances in understanding of these mechanisms, little is known about the role that mitoK\textsubscript{ATP} channel modulation could play in ROS production and functional recovery of the hypoxic-reoxygenated developing heart. Although the embryonic/fetal heart normally grows and functions in a relatively hypoxic environment, it rapidly reacts to oxygen deprivation and reoxygenation, e.g., as in the case of transient uteroplacental ischemia. We previously characterized the chrono-, dromo-, and inotropic responses of the isolated embryonic chick heart (4–5 days old) to anoxia-reoxygenation (30, 34). We also found that this preparation is subjected to an oxidative stress during reoxygenation (28) similar to reperfused cardiac cells obtained from 10-day-old chick embryo (41, 45). Interestingly, the postischemic protection of embryonic ventricular myocytes afforded by mitoK\textsubscript{ATP} channel opening involves ROS and/or NO production (6, 19). On the other hand, we recently found that inducible NO synthase (NOS) is strongly expressed in the embryonic myocardium and generates NO during anoxia-reoxygenation (38), improving recovery of excitation-contraction (E-C) coupling in the ventricle (21). However, the underlying protective mechanism involving the mitoK\textsubscript{ATP} channel remains to be explored in the whole developing heart.

In the present study, we asked whether pharmacological opening of the mitoK\textsubscript{ATP} channel improves postanoxic recovery of pacemaking activity, cell-to-cell communication, myocardial contractility, and E-C coupling. Using a pharmacological approach, we also investigated the signaling pathways operating in the embryonic myocardium, with special emphasis on ROS-, NO-, and PKC-dependent pathways.

MATERIALS AND METHODS

This investigation fully conforms with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society.

Reagents

The fluorescence probe 2',7'-dichlorofluorescin diacetate (DCFH-DA), the mitochondrial complex III inhibitor myxothiazol (MyxO), the radical scavenger N-(2-mercaptopropionyl)glycine (MPG), the NOS inhibitor N\textsuperscript{G}-nitro-l-arginine methyl ester (l-NAME), the mi-

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In Vitro Mounting of the Heart

Fertilized eggs from Lohman Brown hens were incubated for 96 h at 38°C and 95% relative humidity to reach embryonic stage 24HH [according to Hamburger and Hamilton (13)]. The intact and spontaneously beating heart was carefully excised and placed in the culture compartment of an airtight chamber. The chamber was equipped with two windows for observation and measurements and maintained under controlled metabolic conditions on the thermostabilized stage (37°C) of an inverted microscope (model IMT2, Olympus, Tokyo, Japan) as previously described in detail (30, 38). Briefly, the culture compartment (300 µl) was separated from the gas compartment by a thin (15 µm) transparent and gas-permeable silicone membrane (RTV 141, Rhône-Poulenc, Lyon, France). The heart was slightly flattened by the silicone membrane, and the resulting thickness of the myocardial tissue facing the gas compartment was ≈300 µm. Thus PO2 at the tissue level could be strictly controlled and rapidly modified (within <5 s) by flushing high-grade gas of selected composition through the gas compartment. At this developmental stage, the heart lacks vascularization and the myocardial oxygen requirement is met exclusively by diffusion.

The standard HCO3−/CO2−-buffered medium was composed of (in mM) 99.25 NaH2PO4, 10 NaHCO3, 4 KCl, 0.79 MgCl2, 0.75 CaCl2, and 8 d-glucose. This culture medium was equilibrated in the chamber with 2.3% CO2 in air (normoxia and reoxygenation) or in N2 (anoxia), yielding pH 7.4.

MPG (1 mM), 5-HD (500 µM), L-NAME (50 µM), and Myxo (10 µM) were first dissolved in this medium (control), Diazox (50 µM), Glib (1 µM), and Chel (5 µM) were first dissolved in DMSO (vehicle) and then diluted in the medium. The final DMSO concentration was 0.5%.

Measurement of Oxidant Stress

ROS generation was assessed using the nonfluorescent probe 2',7'-dichlorodihydrofluorescein (DCFH). The membrane-permeable diacetate form of DCFH (DCFH-DA) was dissolved in methanol at 40 mM (stock aliquots) and kept at −20°C. Before experimentation, the stock solution was diluted in culture medium to give a final concentration of 10 µM. Within the cell, esterases cleave the acetate groups of DCFH-DA, thus trapping DCFH intracellularly (23). DCFH is known to be preferentially oxidized by H2O2 or hydroxyl radical (OH•) but poorly by superoxide anion (O2•−) (12), yielding the fluorescent oxidized probe 2',7'-dichlorofluorescein (DCF).

Isolated hearts were loaded with DCFH-DA for ~30 min at room temperature in the dark. Then the hearts were mounted in the culture chamber, and the medium was renewed with fresh DCFH-DA solution. The chamber was placed on the thermostabilized stage (37°C) of an epifluorescence microscope (Leitz) 15 min before the first fluorescence measurement. The fluorescence, expressed in arbitrary units, was measured on an aorta (300 µm diameter) every 30 s throughout anoxia-reoxygenation with use of an excitation wavelength of 490 nm and an emission wavelength of 533 ± 3 nm. To avoid photobleaching of the fluorescent dye, illumination time was limited to 6 ms. The slope of the fluorescent signal vs. time represented the rate of production of oxyradicals and was expressed as arbitrary units per second.

To take into consideration interindividual variations in size and slight developmental differences of the hearts, end-diastolic wall area of the ventricle (mm2) was obtained from frames of video recordings of contracting heart obtained at the end of each experiment, as previously described (29). Moreover, the density of protein of the ventricular wall was obtained by dividing the protein content by the area and expressed as micrograms of protein per square millimeter.

Recording of Electrical and Mechanical Contractions

Electrical and contractile activities were recorded simultaneously and continuously throughout in vitro experiments as previously described (30, 34).

Electrical activity. ECG activity of the spontaneously contracting hearts was recorded using two Ag/AgCl electrodes 1 mm apart (0.3 mm diameter) inserted into the window facing the culture compartment. Briefly, the atrial and ventricular regions were placed in the immediate vicinity of these electrodes, which were connected to a differential preamplifier (gain = 2,000), resulting in an output signal of ~1 V from peak to peak. This signal was digitized and processed using an Apple Macintosh computer. The ECG displayed characteristic P, QRS, and T components, which allowed assessment of beating rate (beats/min) and atrioventricular (AV) conduction delay (PR interval). Additionally, the various types of arrhythmias (mainly sinoatrial arrest; brady- and tachycardia; 1st-, 2nd-, and 3rd-degree AV block; and Wenckebach phenomenon) and their duration could be precisely determined.

Contractile activity. Two adjustable phototransistors were positioned over the projected image of the sinoatrial pacemaking region and the apex of the ventricle, allowing detection of edge motion of the myocardial wall. The actual distance between investigated atrial and ventricular regions was the same in all groups: 2.3 ± 0.2 mm (n = 56). The mean propagation velocity of the wave of contraction was obtained by dividing this distance by the time between the peaks of the maximal shortening velocity in atrium and ventricle (i.e., mechanical AV delay).

The actual ventricular shortening at the apex (S, µm) was determined using video recordings obtained before anoxia and at the end of reoxygenation. The maximal shortening and relaxation velocities were obtained from the maximal positive and negative values of the first derivative of S (dS/dt, mm/s), respectively.

E-C coupling. The electromechanical delay (EMD, ms), reflecting the efficiency of E-C coupling, was determined at the level of the atrium (EMDA) and ventricle (EMDV) by measuring the delay between the electrical and mechanical events, i.e., the interval of time between the very initial phase of the P and QRS components and the initiation of contraction in the atrium and ventricle, respectively.

Anoxia-Reoxygenation Protocol

After 45 min of in vitro stabilization under normoxia at 37°C, the hearts were subjected to 30 min of strict anoxia followed by 60 min of reoxygenation. The pharmacological agents were present throughout anoxia-reoxygenation.

Protein and Glycogen Determination

At the end of each experiment, the hearts were carefully dissected into atria, ventricle, and conotruncus and stored at −20°C for subsequent determination of protein and glycogen content, as described previously (20).

Statistical Analysis

Values are means ± SD, unless otherwise indicated. The significance of any difference between the groups was assessed using Student’s t-test or analysis of variance (ANOVA) with Tukey’s post hoc test. The differences between the rates of functional recovery in the experimental groups were assessed by repeated-measures ANOVA during the first 20 min of reoxygenation. To take into consideration preanoxic variations between groups, all parameters are
RESULTS

Functional Parameters Under Normoxia

Only spontaneously and regularly contracting hearts were used in this study. The preanoxic baseline parameters were not significantly altered by any of the agents used, either alone or in combination (Table 1).

Oxidant Stress in the Anoxic-Reoxygenated Embryonic Heart

Figure 1 shows that the time course of DCF fluorescence intensity measured in the ventricle essentially depended on the presence of oxygen. Preanoxic ROS production was not affected by any of the agents, alone or in combination (Fig. 2). On reoxygenation, the rate of ROS production was maximal after 10 ± 3 min in the control group (n = 6) and was reduced by 73 and 60% in the presence of Myxo and MPG, respectively (P < 0.02; Fig. 2).

Effects of K<sub>ATP</sub> Channel Modulation on ROS Production During Reoxygenation

Opening of the mitoK<sub>ATP</sub> channel by Diazo doubled ROS production with respect to vehicle (P < 0.005). This Diazo-induced ROS production was abolished by 5-HD, MPG, or L-NAME but not by Chel. However, 5-HD, L-NAME, or Chel alone had no effect on ROS production (Fig. 2).

Recovery of AV Conduction and E-C Coupling Was Improved by Activation of the mitoK<sub>ATP</sub> Channel

Although all parameters fully recovered at the end of reoxygenation, opening of the mitoK<sub>ATP</sub> channel with Diazo significantly improved the rate of recovery of the PR interval and EMD<sub>a</sub>, during the first 20 min of reoxygenation (Fig. 3). On the contrary, recovery of the other functional parameters was not affected by Diazo, 5-HD abolished the Diazo-induced protection of EMD<sub>a</sub>, but not that of the PR interval. However, 5-HD alone did not have an effect on the rate of recovery of the functional parameters compared with the control group (Fig. 4). The nonselective K<sub>ATP</sub> channel blocker Glib systematically induced atrial tachycardia during normoxia and reoxygenation associated with frequent second-degree AV blocks (not shown). The mechanical AV delay was 142 ± 22 ms (n = 56) under preanoxia and 227 ± 48 ms (n = 25) at the onset of reoxygenation and displayed the same time course as the electrical AV delay (PR interval), without regard to treatment. Furthermore, the mean propagation velocity of the wave of contraction between the atrium and ventricle passed from 16.6 ± 3.1 mm/s (n = 56) under preanoxia to 10.8 ± 2.5 mm/s (n = 25; P < 0.0001) at the onset of reoxygenation.

Protection of E-C Coupling Was Abolished by ROS Scavenger or PKC Inhibitor

During reoxygenation, treatment with MPG alone decreased ROS production (Fig. 2) and increased EMD<sub>a</sub> (Fig. 4) but had no effect on other functional parameters. The Diazo-induced burst of ROS observed during reoxygenation was significantly lower in the Diazo + MPG group than in the Diazo group and comparable with that of vehicle group (Fig. 2). Moreover, scavenging ROS by MPG abolished the protective effect of Diazo on EMD<sub>a</sub> (Fig. 3). By contrast, heart rate, PR interval, EMD<sub>a</sub>, and ventricular shortening were not altered by the addition of MPG (Fig. 3).

During reoxygenation, Chel did not alter Diazo-induced ROS production (Fig. 2) but abolished the improvement in EMD<sub>a</sub> by the mitoK<sub>ATP</sub> channel opener (Fig. 5). Furthermore, the Wenckebach phenomenon was observed during 52 ± 14 min in the Diazo + Chel group (n = 5) and during 19 ± 11 min (n = 7), 19 ± 9 (n = 7), and 20 ± 4 min (n = 4) in the Diazo, vehicle, and Chel groups, respectively (P < 0.01, Diazo + Chel vs. others). Chel alone did not affect ROS production or functional parameters under steady normoxia or during reoxygenation (Figs. 2 and 5).

Differences in sensitivity to anoxia-reoxygenation between the atrium and ventricle are illustrated by systematical longer EMD<sub>a</sub> than EMD<sub>v</sub> under baseline conditions (Table 1) and no significant effect of reoxygenation on EMD<sub>a</sub> (Figs. 3–6).

Endogenously Produced NO Was Necessary for Diazo-Induced Protection

L-NAME alone did not affect ROS production throughout anoxia-reoxygenation (Fig. 2) but significantly delayed recovery of EMD<sub>a</sub> during reoxygenation (Fig. 4). Furthermore, L-NAME abolished the Diazo-induced ROS production (Fig.

Table 1. Baseline functional parameters were not altered by any of the treatments

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 6)</th>
<th>5-HD (n = 5)</th>
<th>L-NAME (n = 4)</th>
<th>MPG (n = 4)</th>
<th>Vehicle (n = 7)</th>
<th>Diazo (n = 7)</th>
<th>Diazo + 5-HD (n = 5)</th>
<th>Diazo + MPG (n = 4)</th>
<th>Diazo + Chel (n = 5)</th>
<th>Diazo + L-NAME (n = 4)</th>
<th>Chel (n = 4)</th>
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<tr>
<td>Heart rate, beats/min</td>
<td>151 ± 24</td>
<td>160 ± 8</td>
<td>162 ± 12</td>
<td>132 ± 5</td>
<td>168 ± 12</td>
<td>177 ± 34</td>
<td>160 ± 6</td>
<td>143 ± 18</td>
<td>144 ± 7</td>
<td>161 ± 13</td>
<td>164 ± 13</td>
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<td>PR interval, ms</td>
<td>133 ± 25</td>
<td>134 ± 28</td>
<td>145 ± 20</td>
<td>129 ± 19</td>
<td>122 ± 6</td>
<td>118 ± 15</td>
<td>129 ± 15</td>
<td>132 ± 15</td>
<td>142 ± 10</td>
<td>125 ± 25</td>
<td>134 ± 26</td>
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<tr>
<td>Mechanical AV delay, ms</td>
<td>143 ± 26</td>
<td>141 ± 28</td>
<td>159 ± 19</td>
<td>140 ± 13</td>
<td>130 ± 13</td>
<td>126 ± 14</td>
<td>136 ± 19</td>
<td>139 ± 19</td>
<td>158 ± 7</td>
<td>139 ± 28</td>
<td>146 ± 25</td>
</tr>
<tr>
<td>EMD&lt;sub&gt;a&lt;/sub&gt;, ms</td>
<td>16 ± 4</td>
<td>14 ± 3</td>
<td>18 ± 3</td>
<td>16 ± 3</td>
<td>13 ± 2</td>
<td>13 ± 4</td>
<td>15 ± 4</td>
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<td>14 ± 2</td>
<td>21 ± 7</td>
<td>15 ± 0</td>
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<tr>
<td>EMD&lt;sub&gt;v&lt;/sub&gt;, ms</td>
<td>10 ± 3</td>
<td>7 ± 3</td>
<td>13 ± 3</td>
<td>10 ± 4</td>
<td>10 ± 2</td>
<td>12 ± 5</td>
<td>11 ± 1</td>
<td>10 ± 7</td>
<td>12 ± 6</td>
<td>12 ± 3</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>Ventricular shortening, μm</td>
<td>22 ± 21</td>
<td>27 ± 16</td>
<td>7 ± 3</td>
<td>13 ± 7</td>
<td>28 ± 21</td>
<td>29 ± 22</td>
<td>28 ± 27</td>
<td>8 ± 4</td>
<td>7 ± 4</td>
<td>17 ± 12</td>
<td>8 ± 5</td>
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<tr>
<td>Ventricular shortening velocity, mm/s</td>
<td>1.5 ± 1.3</td>
<td>1.4 ± 0.6</td>
<td>0.6 ± 0.3</td>
<td>0.9 ± 0.4</td>
<td>2.1 ± 1.6</td>
<td>2.0 ± 1.5</td>
<td>2.0 ± 1.9</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>0.6 ± 0.3</td>
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Values are mean ± SD; n, number of hearts. AV, atrioventricular; EMD<sub>a</sub> and EMD<sub>v</sub>, atrial and ventricular electromechanical delay, respectively; 5-HD, 5-hydroxydecanoate; L-NAME, N<sub>0</sub>-nitro-l-arginine methyl ester; MPG, N-(2-mercaptopropionyl)glycine; Diazo, diazoxide; Chel, chelerythrin. There was no significant difference between groups (ANOVA with Tukey’s test).
2) and protection of AV conduction and EMD, during reoxygenation (Fig. 6). Contractile recovery was improved by L-NAME, alone and in combination with Diazo (Figs. 4 and 6).

**Metabolic and Morphometric Parameters**

The protein content of the whole heart, atria, ventricle, and conotruncus was 69.7 ± 14.3, 15.5 ± 5.4, 42.7 ± 9.1, and 11.1 ± 3.7 µg (n = 109), respectively, and did not vary from one group to another. Glycogen content was 1.2 ± 0.6, 0.7 ± 0.3, and 0.5 ± 0.3 nmol glucose unit/µg protein (n = 109) in atrium, ventricle, and conotruncus, respectively. In hearts on which fluorescence was measured, protein content, area, and density of protein of the ventricular wall were 44.7 ± 7.7, 3.8 ± 0.8 mm², and 11.9 ± 2.9 µg/mm² (n = 45), respectively, with no statistically significant differences between the groups.

**DISCUSSION**

Taken together, our main findings indicate that 1) mitoK<sub>ATP</sub> channel opening by Diazo increases ROS production at reoxygenation exclusively, 2) ROS production is necessary to improve recovery of E-C coupling in the ventricle, 3) this beneficial effect is mediated by PKC, and 4) the Diazo-induced protection requires NOS activation.

**Limitations of the Experimental Model**

Detection of oxidant stress by DCFH fluorescence. Under our experimental conditions, the fluorescence measured in the ventricle was not influenced by the size of the hearts, because the protein density of the ventricular wall did not vary from one group to another. The decrease in fluorescence intensity observed during anoxia might be attributed to the unavoidable diffusion of the accumulated oxidized form of DCFH out of the cell (15). The complex kinetics underlying this backdiffusion are technically difficult to determine, and the changes in DCF fluorescence intensity vs. time (arbitrary units/s) might be slightly underestimated in all experiments to the same extent. The fact that the rate of DCFH oxidation in the absence of oxygen was negligible showed that only oxygen-derived radicals were able to alter fluorescence and that there was no illumination-induced DCFH autoxidation under our experimental conditions (Fig. 1).

Although the vehicle DMSO used in this study is also known as a cell-penetrating OH· scavenger, it affected neither ROS production nor the time course of functional parameters with respect to the vehicle-free control condition.

**Contractile activity.** In contrast to the chronotropic and dromotropic parameters, the ventricular shortening and maxi-

Fig. 1. Representative recording of 2',7'-dichlorofluorescein (DCF) fluorescence intensity in ventricle of 4-day-old embryonic chick heart under steady anoxia (0% O₂; a), under steady normoxia (21% O₂; b), and during an episode of normoxia-anoxia-reoxygenation (c). Rate of reactive oxygen species (ROS) production, expressed as arbitrary units (au) per second, was systematically determined after stabilization under normoxia (open arrow) and during reoxygenation-induced oxidative burst (solid arrow). Inset: scanning-electron-microscopic image of the heart at stage 24 HH showing the investigated circular region (dashed line). LA and RA, left and right atrium; AV, atrioventricular canal; V, ventricle; CT, conotruncus.

Fig. 2. Rate of ROS production determined using DCF fluorescence at the level of the ventricle under steady preanoxia (A) and during reoxygenation (B) after 30 min of anoxia in control hearts and hearts treated with myxothiazol (Myxo), N-(2-mercaptobenzyl)glycine (MPG), N<sub>N</sub>-nitro-L-arginine methyl ester (l-NAME), 5-hydroxydecanoate (5-HD), diazoxide (Diazo), and chelerythrine (Chel), alone or in combination. Solid bars, no vehicle (control); shaded bars, DMSO (vehicle). Values are means ± SE of number of hearts in parentheses. Statistical significance is as follows: §P < 0.02 vs. control; *P < 0.003 vs. vehicle; †P < 0.01 vs. Diazo (Student’s t-test).
mal shortening velocity displayed important interindividual variations, even after in vitro stabilization (Table 1). Such variability of contractile activity might be due to slight differences in the developmental stage, variations of cardiac three-

Fig. 3. Functional parameters during anoxia-reoxygenation as percent changes of their preanoxic values. Mitochondrial ATP-sensitive K⁺ (mitoK<sub>ATP</sub>) channel opener Diazo did not affect recovery of heart rate, ventricular shortening, or atrial electromechanical delay (EMDa) but improved recovery of AV conduction (PR interval) and ventricular electromechanical delay (EMDv). Protection of EMDv was abolished by the mitoK<sub>ATP</sub> channel blocker 5-HD (Diazo + 5-HD) or by the antioxidant MPG (Diazo + MPG). Values are means ± SE of number of hearts in parentheses. *P < 0.05, Diazo vs. vehicle; **P < 0.05, Diazo vs. vehicle, Diazo + 5-HD, or Diazo + MPG (repeated-measures ANOVA during the first 20 min of reoxygenation).

Fig. 4. During reoxygenation, 5-HD or MPG alone did not have an effect on functional parameters, whereas L-NAME alone delayed EMDv recovery and improved shortening. Values (means ± SE of number of hearts in parentheses) are expressed as percentage of preanoxic values. *P < 0.05, L-NAME vs. control; **P < 0.05, MPG vs. control (repeated-measures ANOVA during the first 20 min of reoxygenation).
dimensional structure, and/or degree of flattening of the hearts in the culture compartment, as discussed previously (29, 30). Shortening determined just before anoxia (19 ± 17 μm, n = 56) and at the end of reoxygenation (20 ± 16 μm, n = 56), as well as the rate of contractile recovery, was similar in all experimental groups, indicating that contractile activity fully recovered. Furthermore, inotropy and lusitropy were altered to the same extent, because the ratio of the maximal contraction...
velocity to the maximal relaxation velocity was stable throughout anoxia-reoxygenation: 1.25 ± 0.25 (n = 56).

Opening of the mitoK<sub>ATP</sub> Channel Increases ROS Production and Improves Functional Recovery

The maximal rate of ROS production was four times higher during reoxygenation than during preanoxia, indicating that the embryonic heart is subjected to an important postanoxic oxidative stress. This is corroborated by the 60–70% reduction in ROS production by MPG or Myxo, indicating also that mitochondrial complex III was the main source of ROS during reoxygenation.

It has been shown that treatment of adult human and mammalian cardiomyocytes with the mitoK<sub>ATP</sub> channel opener Diazo before, but not during, ischemia-reperfusion increased ROS production and mimicked ischemic preconditioning (3, 7, 25, 31). However, in our experimental model, Diazo was present before and during anoxia-reoxygenation and increased ROS production during the early phase of reoxygenation, but not during normoxia. These differences between the adult and developing heart may be partly attributed to the degree of mitochondrial differentiation (35, 43) illustrated by a low oxidative metabolism (29) and to a limited antioxidant capacity at the stage investigated. Moreover, the fact that 5-HD alone affected neither ROS production nor functional recovery suggests that the mitoK<sub>ATP</sub> channel remains closed throughout anoxia-reoxygenation in the control hearts. Interestingly, the Diazo-induced additional ROS production was associated with an improved recovery of ventricular E-C coupling during reoxygenation. The facts that Diazo is 1,000-fold more potent to activate mitoK<sub>ATP</sub> than sarcolemmal K<sub>ATP</sub> channels (9) and that the nonselective K<sub>ATP</sub> channel blocker Glib, but not 5-HD, induced major atrial arrhythmias associated with second-degree AV blocks clearly indicate that the mitoK<sub>ATP</sub> channels are directly involved in Diazodependent protection of E-C coupling, whereas sarcolemmal K<sub>ATP</sub> channels play no significant role. The obvious proarrhythmic effect of Glib observed in this work suggests, however, that the sarcolemmal K<sub>ATP</sub> channels might play a crucial role in controlling the membrane potential, especially in the immature sinoatrial pacemaker cells.

The fact that MPG abolished Diazodependent ROS production and E-C coupling protection during reoxygenation clearly indicates that ROS are also directly involved in the protective effect of Diazode in the embryonic heart. These results are in agreement with recent reports on pharmacological preconditioning using mitoK<sub>ATP</sub> opener in adult mammalian (7, 27), adult human (3), and embryonic chick (19) cardiomyocytes. Thus the postanoxic protection of E-C coupling afforded by mitoK<sub>ATP</sub> channel opening seems to be mediated by an additional ROS production, independent of the investigated species, the experimental models, or the selected protocol.

PKC Protects Selectively Ventricular E-C Coupling

During reoxygenation, Chel abolished the Diazodependent protection of EMDv, suggesting that E-C coupling is modulated by PKC, which can be indirectly activated by mitoK<sub>ATP</sub> channel opening via ROS production (Fig. 7). The E-C coupling machinery (mainly the plasmalemmal L-type Ca<sup>2+</sup> channel, the ryanodine receptor channel, SERCA2, and phospholamban) is present and starts to be functional in the 4-day-old embryonic chick heart (37). It has previously been found that Ca<sup>2+</sup> entry through a sarcolemmal L-type Ca<sup>2+</sup> channel predominately contributes to the Ca<sup>2+</sup> transient during the cardiac cycle and strongly contributes to E-C coupling (39) but also participates in reoxygenation-induced Ca<sup>2+</sup> overload and myocardial dysfunction at stage 24HH (37). These observations further strengthen the important role that L-type Ca<sup>2+</sup> channel modulation by PKC may play in postanoxic recovery of the developing heart. Indeed, although controversial (17), PKC activity has been reported to restore Ca<sup>2+</sup> homeostasis and play an important role in regulation of E-C coupling during postischemic reperfusion, whereas Chel, at the concentration used in the present study, significantly enhances postischemic Ca<sup>2+</sup> overload in the adult heart (36). On the basis of our findings, we propose that such mechanisms operate also in the anoxoreoxygenated embryonic heart. However, the role of different PKC isoforms, as well as their target proteins, deserves further investigation.

Moreover, the fact that Chel did not affect Diazodependent ROS production but abolished the protection of EMDv afforded by Diazo during reoxygenation suggests that PKC activation is downstream of Diazodependent ROS in the embryonic heart (Fig. 7).

Endogenous NO Plays an Important Role in Diazodependent ROS Production and Cardioprotection

Recently, it has been shown that exogenous NO can activate the mitoK<sub>ATP</sub> channel (33) and that NO and ROS are necessary

![Fig. 7. Schematic representation based on the present findings and illustrating interrelations between mitoK<sub>ATP</sub> channel, cytosolic and mitochondrial nitric oxide synthases (NOS and mitNOS, respectively), diazoxide-induced mitochondria-derived reactive oxygen species (ROS), and PKC during postanoxic reoxygenation of the embryonic heart. For the sake of clarity, only the major effects of NO, ROS, and PKC on recovery of cell-to-cell conduction through gap junctions (gap j) and ventricular excitation-contraction (E-C) coupling during reoxygenation are indicated. Arrow, activation; T-shaped symbol, inhibition. Pharmacological agents are indicated in italics. Cx, connexins; L-type Ca</sup>sup>2+</sup>, L-type Ca</sup>sup>2+</sup> channel; RyR, ryanodine receptor channel; SR, sarcoplasmic reticulum; I-IV, respiratory chain complexes; sm, sarcolemmal membrane; im and om, inner and outer mitochondrial membranes, respectively.](http://ajpheart.physiology.org/)[/raw]
to increase cell survival of 10-day-old chick cardiomyocytes via opening of mitoK_ATP channels (19). We recently showed that the embryonic heart produces NO during anoxia-reoxygenation mainly derived from inducible NOS, which is known to be strongly expressed early during cardiogenesis (38). The present data show that l-NAME alone delays recovery of EMD, as in our previous work (21). These findings are in agreement with studies showing that NO can increase the open probability of the ryanodine receptor channel (46), which could accelerate Ca^{2+} release from the sarcoplasmic reticulum and, thus, shorten EMD, during reoxygenation (Fig. 7). The fact that NOS inhibition had no effect on the DCF signal during anoxia-reoxygenation indicates that production of ONOO− resulting from a possible reaction between NO and O_2^- is negligible in the embryonic heart under control conditions. Indeed, the DCFH technique is known to be sensitive not only to H_2O_2 and OH• but also to ONOO− (15). Moreover, myocardial levels of nitration of protein tyrosine by ONOO− (nitrotyrosine assessed by immunoblotting assay) were not increased after anoxia-reoxygenation (Raddatz and Markert, unpublished data).

Because l-NAME abolished Diazoxide-mediated ROS production and functional protection, activation of NOS appears to be a prerequisite for the beneficial effects of mitoK_ATP channel opening in the setting of anoxia-reoxygenation. It seems that cardiac mitochondria contain an NOS isoform (mtNOS) (18, 48) and that NO inhibits mitochondrial respiration at the complex IV level (2) and increases H_2O_2 production (32). On the basis of these observations and our findings, we hypothesize that endogenously produced NO could act directly on proteins involved in E-C coupling when mitoK_ATP channels are closed, as under control conditions. On the other hand, opening of the mitoK_ATP channel by Diaz could activate mtNOS and increase intramitochondrial NO production, resulting in increased ROS production at the level of complex III and I. This additional ROS production could activate PKC and result in phosphorylation of the L-type Ca^{2+} channel and/or ryanodine receptor channel, improving functional recovery (Fig. 7).

Our data showing that the PR interval was significantly prolonged and that the incidence of AV blocks was specially high during anoxia and reoxygenation clearly indicate that cell-to-cell communication was strongly altered. Indeed, although there is no specialized conduction system, various connexins (e.g., Cx43) are expressed and functional during early cardiogenesis, and their relative abundance (44), electrophysiological characteristics (4, 42), and regulation (5) vary throughout development. The fact that Diazoxide-induced protection of the PR interval was not suppressed by any of the agents, except l-NAME, suggests that NO, associated with Diazoxid, is necessary to protect cell-to-cell communication during anoxia-reoxygenation. Indeed, it has been shown that electrical uncoupling of cardiomyocytes during ischemia can be accelerated by K_ATP channel inhibition (16) and that l-NAME can decrease Cx43 phosphorylation and expression in vascular muscle (11), modulating cell-to-cell communication. However, information about a possible interaction of NO and Diazoxid with connexins is not available in the literature, and the exact mechanisms underlying this protection remain to be explored.

In conclusion, to the best of our knowledge, this study is the first to explore reoxygenation-induced alterations of electro-mechanical coupling in relation to pharmacological modulation of mitoK_ATP channels in the developing heart. Our results show that activation of the mitoK_ATP channel by Diazoxide throughout anoxia-reoxygenation improves recovery of AV conduction and ventricular E-C coupling without significantly affecting other parameters. Furthermore, protection of E-C coupling by mitoK_ATP channel opening requires NO activation, ROS signaling, and PKC-sensitive pathways.

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REFERENCES

19. Lebuffe G, Schumacker PT, Shao ZH, Anderson T, Iwase H, and Vanden Hoek TL. ROS and NO trigger early preconditioning: relationship-


