Effects of Cardioplegic Arrest and Reperfusion on Rabbit Cardiac Ryanodine Receptors

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Calcium overload is considered to be a primary contributor to ischemia–reperfusion injury. Cardiac sarcoplasmic reticulum (SR), the main regulator of intracellular Ca\(^{2+}\) concentration under normal conditions, is a target for ischemic myocardial injury. The ryanodine receptor (RyR) is the SR Ca\(^{2+}\) release channel. Previous reports have shown that a reduction in RyR activity during global myocardial ischemia correlates with concomitant myocardial dysfunction. Crystalloid cardioplegia, a technique for myocardial protection during heart operations, reduces Ca\(^{2+}\) accumulation during global ischemia. Hence, the effects of cardioplegia on RyR in isolated rabbit hearts was investigated. The study also compared \(^{3}\)H\) ryanodine binding before ischemia (control group), after 30 min of ischemia (either global ischemia (GI group) or cardioplegic arrest (CA group)), and after 20 min of reperfusion. The GI group, but not the CA group, showed a significant reduction in the maximum number of binding sites (Bmax) for RyR compared with the control group (Control vs GI group: after ischemia, 1.33±0.27 vs 0.83±0.12 pmol/mg protein; p<0.05; after reperfusion, 1.33±0.27 vs 0.80±0.08 pmol/mg protein; p<0.05). CA group: after ischemia, 1.22±0.20 pmol/mg protein; after reperfusion, 1.15±0.28 pmol/mg protein). The affinity (Kd) values for \(^{3}\)H\) ryanodine binding were not different among the 3 groups at any point. The preservation of RyR numbers during cardioplegia correlated with the concomitant preservation of cardiac functions. The results indicate that number of functional RyR was much better preserved during cardioplegia than during global ischemia. It is postulated that cardioplegia-induced protection of cardiac RyR may result in the protection of SR function during ischemia–reperfusion.  

Key Words: Cardioplegia; Ischemia–reperfusion injury; Ryanodine receptors

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Myocardial contraction and relaxation are regulated by intracellular Ca\(^{2+}\) concentration, and cardiac sarcoplasmic reticulum (SR) plays an important role in Ca\(^{2+}\) uptake, storage, and release. Under normal conditions, the entry of extracellular Ca\(^{2+}\) via L-type Ca\(^{2+}\) channels in the sarcolemma of the myocardium triggers the release of Ca\(^{2+}\) from the SR, leading to myocardial contraction. Conversely, Ca\(^{2+}\) uptake by the SR results in myocardial relaxation. The SR contains 2 important constituents, Ca\(^{2+}\)-ATPase and the ryanodine receptor (RyR). Ryodine receptor is the Ca\(^{2+}\) release channel on which ryanodine has one of 2 effects, depending on its concentration. Low concentrations of ryanodine bind to the high-affinity site and open the Ca\(^{2+}\) channel; high concentrations of ryanodine close the Ca\(^{2+}\) channel.

During global ischemia, intracellular Ca\(^{2+}\) is increased (the so-called ‘Ca\(^{2+}\) overload’), which leads to myocardial dysfunction. In this situation, the Na\(^{+}\)–Ca\(^{2+}\) exchanger, RyR, and/or SR Ca\(^{2+}\)-ATPase play important roles in maintaining intracellular Ca\(^{2+}\) homeostasis. Such Ca\(^{2+}\) overload is considered to be a primary contributor to ischemia–reperfusion injury.

During ischemia, the SR is one of the targets for ischemic myocardial injury. A number of reports indicate that ischemia depresses SR function; however, both the nature and cause of this effect remain controversial. Darling and coworkers reported that the maximum number of binding sites (Bmax) for ryanodine binding to isolated dog SR vesicles was reduced after 60 min of ischemia. In addition, Zucchi and colleagues noted that in rat hearts the Bmax for high-affinity ryanodine binding to an enriched fraction of SR vesicles was reduced after 20 min of ischemia, and that the reduction persisted after reperfusion. These investigators concluded that the number of RyR was reduced after ischemia, and that this effect might contribute to an alteration in SR function. Using a mature rabbit heart homograft, Matsuda and colleagues observed that while the Bmax for high-affinity ryanodine binding was reduced after 20 min of ischemia, there was no difference between ischemia and non-ischemia in the expression levels of RyR mRNA or protein.

Cold crystalloid cardioplegia is a widely used technique for myocardial protection during heart operations. Its effectiveness in myocyte protection has been well established. The observation that intracellular Ca\(^{2+}\) accumulation in the myocardium is attenuated during an arrest produced by the potassium/magnesium cardioplegia suggests that cardioplegia might act on the SR. However, the effects of cardioplegia on RyR remain unknown.

The present study was designed to investigate the effects of cold crystalloid cardioplegia (potassium/magnesium cardioplegia) on RyR in isolated rabbit hearts during ischemia.
and reperfusion.

Materials and Methods

Surgical Procedure

Japanese white rabbits weighing 1.90–2.65 kg were anesthetized with 0.7 ml/kg ketamine injected intramuscularly and 10 mg/kg sodium pentobarbital injected through the marginal ear vein. The lungs were ventilated with 100% oxygen via a volume-controlled ventilator. Anesthesia was maintained with 1.0–2.0% halothane. The thorax was opened and 1,000 U of heparin was given intravenously. After 30 s, the heart was excised and immersed in ice-cold saline. The aorta was cannulated, a venting tube was placed through the apex, and a latex balloon mounted on a fluid-filled catheter, which was positioned in the left ventricle through the mitral annulus for measuring intracavity pressure. The heart was perfused in a retrograde fashion with modified Krebs–Henseleit bicarbonate buffer solution consisting of 119.0 mmol/L NaCl, 25.0 mmol/L NaHCO3, 4.6 mmol/L KCl, 1.2 mmol/L KH2PO4, 1.2 mmol/L MgSO4, 1.3 mmol/L CaCl2, and 11.0 mmol/L glucose (pH 7.4; 37°C) at a pressure of 80 cm H2O. The buffer was equilibrated with 95% oxygen and 5% carbon dioxide.

All rabbits received humane care in compliance with the Guide for the Care and Use of Laboratory Animals.14

Experimental Protocols

The experimental protocols are shown in Fig 1. The animals were divided into 3 groups. In the control group (n=5), after 20 min of controlled perfusion with oxygenated Krebs–Henseleit bicarbonate buffer solution, basal measurements of hemodynamic variables were taken immediately. Then, the myocardium was sampled for the ryanodine-binding assay. The global ischemic group (GI group; n=11) also underwent 20 min of controlled perfusion, but each heart was then subjected to 30 min global ischemia at room temperature by clamping the aortic cannula, followed by 20 min of reperfusion. The myocardium was sampled for ryanodine-binding assay after ischemia and after reperfusion. In the cardioplegic arrest group (CA group; n=9) each heart underwent 20 min of controlled perfusion, 30 min of cardioplegic arrest, and 20 min of reperfusion. In each heart in the CA group, 10 ml/kg body weight of the cold crystalloid cardioplegic solution was administered via the aortic cannula at a perfusion pressure of 80 cm H2O. The cardioplegic solution contained 85.3 mmol/L Na, 25.0 mmol/L K, 0.95 mmol/L CaCl2, 0.95 mmol/L EGTA (20 mmol/L free Ca2+), and 0.95 mmol/L EGTA (20 mmol/L free Ca2+), in each case with a concentration of [3H] ryanodine from within the range 0.6–20 nmol/L. The reaction was terminated by rapid filtration of 1 ml of the incubation mixture through a Millipore filter (type HA, pore size 0.45 μm) under reduced pressure. To minimize the non-specific binding component, each filter was immediately washed with 5 ml of ice-cold buffer (25 mmol/L imidasole, 1.0 mol/L KCl, 1.103 mmol/L CaCl2, and 0.95 mmol/L EGTA, at pH 7.4), then removed while under vacuum. After the addition of 5 ml of scintillation fluid, the radioactivity was counted in a scintillation counter (LSC-5100; Aloka, Tokyo, Japan). Non-specific binding was determined in the presence of 2 μmol/L unlabeled ryanodine. The procedures described earlier were performed for the control group (n=5), GI group (after ischemia, n=5; after reperfusion, n=5), and the CA group (after ischemia, n=5; after reperfusion, n=4).

Preparation of Sarcoplasmic Reticulum Membranes

Sarcoplasmic reticulum membranes from rabbit ventricular muscle were prepared using the method described by Kranias and coworkers.15 After excision, each heart was cut into pieces with scissors in ice-cold 0.9% NaCl and rinsed thoroughly to remove all traces of blood. The whole ventricles were homogenized in 30 mmol/L Tris-maleate buffer at pH 7.0 containing 0.3 mol/L sucrose, 0.6 mol/L KCl, 5 mg/L leupeptin, and 0.1 mmol/L phenylmethyl sulfonyl fluoride (PMSF), at pH 7.0 (solution A) using a Brinkmann Polytron. The homogenate was centrifuged at 5,500 × g for 10 min, and the resulting supernatant was filtered through 4 layers of cheesecloth before centrifugation at 12,000 × g for 20 min. The supernatant was again filtered through cheesecloth and centrifuged at 143,000 × g for 30 min. The pellet was resuspended in a buffer of the following composition: 30 mmol/L Tris-maleate containing 0.3 mol/L sucrose, 0.6 mol/L KCl, 5 mg/L leupeptin, and 0.1 mmol/L PMSF, at pH 7.0 (solution B). This suspension was centrifuged at 143,000 × g for 45 min. The pellet was resuspended in solution B, homogenized, and centrifuged at 143,000 × g, as described earlier. The resulting pellet was also suspended in solution B and centrifuged at 143,000 × g. The final pellet was the microsomal fraction that was rich in SR vesicles, and it was suspended at a concentration of 8–10 mg protein/ml in a buffer of the following composition: 20 mmol/L Tris-maleate containing 0.3 mol/L sucrose, 0.1 mol/L KCl, 5 mg leupeptin and 0.1 mmol/L PMSF, at pH 7.0 (solution C). This fraction was rapidly frozen in liquid nitrogen and stored at −80°C. An aliquot was retained for protein assay, for which the method of Lowry et al16 was used with bovine serum albumin as the standard.

Assay of [3H]Ryanodine Binding

[3H]Ryanodine-binding assays were carried out according to the methods described elsewhere.17,18 Briefly, the microsomal fraction that was rich in SR vesicles (0.4 mg/ml) was incubated for 90 min at 37°C in 25 mmol/L imidasole (pH 7.4), 1.0 mol/L KCl, 1.103 mmol/L CaCl2, and 0.95 mmol/L EGTA (20 mmol/L free Ca2+), in each case with a concentration of [3H] ryanodine from within the range 0.6–20 nmol/L. The reaction was terminated by rapid filtration of 1 ml of the incubation mixture through a Millipore filter (type HA, pore size 0.45 μm) under reduced pressure. To minimize the non-specific binding component, each filter was immediately washed with 5 ml of ice-cold buffer (25 mmol/L imidasole, 1.0 mol/L KCl, 1.103 mmol/L CaCl2, 0.95 mmol/L EGTA, at pH 7.4), then removed while under vacuum. After the addition of 5 ml of scintillation fluid, the radioactivity was counted in a scintillation counter (LSC-5100; Aloka, Tokyo, Japan). Non-specific binding was determined in the presence of 2 μmol/L unlabeled ryanodine. The procedures described earlier were performed for the control group (n=5), GI group (after ischemia, n=5; after reperfusion, n=5), and the CA group (after ischemia, n=5; after reperfusion, n=4).

Western Blot

The microsomal fraction that was rich in SR vesicles (20 μg protein/lane) from the control (n=4), GI (after reperfusion, n=4), and CA (after reperfusion, n=4) groups was electrophoresed on 4% sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE). The proteins in the gel were transferred to a Protran Nitrocellulose Membrane (Schleicher and Schuell, Dassel, Germany). This was immersed in phos-
phate buffer containing non-fat dry milk, incubated with a monoclonal anti-RyR antibody solution (MA3-925; Affinity BioReagents Inc, Golden, CO, USA), and incubated further with peroxidase-conjugated secondary antibody (1:1,000 dilution). The amount of protein recognized by the antibodies was quantified by means of an ECL immunoblotting detection system (Amersham Pharmacia Biotech Inc, NJ, USA), the membrane being exposed to X-ray films. Quantitative densitometry of Western blots was analyzed using a microcomputer imaging device (AE-6900M; ATTO, Tokyo, Japan).

**Hemodynamics**

After 20 min of controlled perfusion, baseline measurements of isovolumic left ventricular function were taken in the GI and CA groups. Left ventricular pressure was measured using a Statham P23ID pressure transducer and recorded on a strip chart (Polygraph; Nihon Denki Sanei, Tokyo, Japan). The left ventricular developed pressure (LVDP) and the positive first derivative of ventricular pressure (+dP/dt) were recorded when the left ventricular end-diastolic pressure was zero. The postischemic LVDP and +dP/dt were measured after 20 min of reperfusion under the same conditions as those used for the baseline measurements. The effects on LV function were assessed by means of the following formula:

\[
\text{Percentage recovery} \, (\%) = \frac{\text{Level after reperfusion}}{\text{Baseline level}} \times 100.
\]

**Statistical Analysis**

Data are expressed as the mean ± SD. Differences between data were analyzed using a one-way analysis of variance followed by Scheffe’s test. Differences were taken to be significant if the p value <0.05.

**Results**

**Assay of [3H] Ryanodine Binding**

Fig 2 shows representative data for high-affinity [3H] ryanodine binding to the SR vesicles prepared from the hearts of the control, GI, and CA groups. Table 1 shows the

**Table 1 [3H] Ryanodine Binding Assay Data**

<table>
<thead>
<tr>
<th></th>
<th>Pre-ischemia</th>
<th>Post-ischemia</th>
<th>Post-reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bmax (pmol/mg)</td>
<td>Kd (nmol/L)</td>
<td>Bmax (pmol/mg)</td>
</tr>
<tr>
<td>Control (n=5)</td>
<td>1.33±0.27</td>
<td>0.90±0.28</td>
<td>0.83±0.12*</td>
</tr>
<tr>
<td>GI group (n=10)</td>
<td></td>
<td></td>
<td>1.22±0.20</td>
</tr>
<tr>
<td>CA group (n=9)</td>
<td>1.22±0.20</td>
<td>1.01±0.23</td>
<td>1.15±0.28</td>
</tr>
</tbody>
</table>

Bmax, maximal number of binding sites; Kd, dissociation constant; GI, global ischemia; CA, cardioplegic arrest. *p<0.05 vs control.
Effects of Cardioplegic Arrest and Reperfusion

Table 2 Hemodynamic Data

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-ischemia</th>
<th>Post-reperfusion</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (beats/min)</td>
<td>LVDP (mmHg)</td>
<td>dP/dt (mmHg/s)</td>
</tr>
<tr>
<td>GI (n=5)</td>
<td>216±44</td>
<td>60.9±11.9</td>
<td>112.0±275</td>
</tr>
<tr>
<td>CA (n=4)</td>
<td>195±39</td>
<td>77.3±22.5</td>
<td>1137±292</td>
</tr>
</tbody>
</table>

HR, heart rate; LVDP, left ventricular developed pressure; +dP/dt, positive first derivative of ventricular pressure; GI, global ischemia; CA, cardioplegic arrest. *p<0.05 vs corresponding GI group value.

mean values obtained for the Bmax and the dissociation constant (Kd) for RyR in all 3 groups. By comparison with the control group, the GI group showed a significant reduction in the Bmax for RyR at the posts ischemia and postreperfusion sampling points. However, the CA group showed no significant reduction in the Bmax for RyR when compared with the control group. The Kd for [3H] ryanodine binding was not significantly different among the 3 groups at any point.

Western Blot Analysis

Western blot was performed in all 3 groups to assess the content of RyR protein in the microsomal fraction that was rich in SR vesicles. Fig.3 shows representative Western blot analysis for each group. Semiquantitative analysis based on densitometry of the Western blots indicates that no significant differences in RyR protein levels were present among the 3 groups.

Hemodynamic Data

Table 2 shows functional parameters for the GI and CA groups. The LVDP, +dP/dt, and %LVDP were all significantly larger in the CA group than in the GI group, whereas heart rate (HR) and %+dP/dt were not significantly different between these 2 groups. Reperfusion arrhythmia (ventricular tachycardia or fibrillation) occurred in 33% of the GI group (2/6 patients). Within the GI group, the occurrence of arrhythmias was not associated with differences in [3H] ryanodine binding.

Discussion

In the present study, we examined the alterations in cardiac function and the SR Ca²⁺ release channels RyR occurring during normothermic global ischemia or cardioplegic arrest achieved by means of cold crystalloid cardioplegia (potassium/magnesium cardioplegia), and subsequent reperfusion. In this study: (i) the number of high-affinity ryanodine binding sites was significantly reduced after 30 min of global ischemia and did not recover during 20 min of reperfusion; (ii) the reduction in the number of high-affinity ryanodine binding sites was smaller during cardioplegic arrest and reperfusion; (iii) the reduction in the number of high-affinity ryanodine binding sites correlated well with the degree of cardiac dysfunction seen in the 2 experimental groups; and (iv) the differences in the expression levels of RyR protein between cardioplegic-arrested hearts and ischemic hearts were not significant.

[3H] Ryanodine binding is a proportionate indicator of the number of channel molecules and is also dependent on the functional structure of the receptors. This being so, the reduction in ryanodine binding seen in the GI group indicates a reduction in Ca²⁺ efflux from the SR because of a decreased opening probability during 30 min of ischemia. This finding is in agreement with previous studies.6,7,8,10 During et al observed a reduction in the Bmax for ryanodine after 60 min of ischemia, but could not conclude that the density of SR channels was affected by ischemia because of contamination by myofilibrillar protein.6 However, Zacchi et al reported that myofilibrillar contamination, as estimated by K-ATPase activity, was not different in SR preparations obtained from controls or after 20 min of ischemia.6 In the present study, we purified the enriched fraction of the SR vesicles for the binding assay because a previous study showed that this successfully avoided contamination by myofilibrillar proteins. The smaller reduction in ryanodine binding seen in the CA group indicated that the number of receptors in the functional state was much better preserved during cardioplegic arrest and reperfusion than during global ischemia–reperfusion. Interestingly, however, no differences in the expression levels of the RyR protein were seen among the control hearts, cardioplegic-arrested hearts, and ischemic hearts. These results indicate that conformational changes may occur during ischemia, resulting in a decrease in ryanodine binding. Matsuda and colleagues reported that although there was a reduction in ryanodine binding, there were no differences in the expression levels of RyR mRNA or protein between the control hearts and ischemic hearts.10 They suggested that global ischemia is associated only with a downregulation of ryanodine receptor activity.

Cardioplegia has been reported to be beneficial both in laboratory experiments and in clinical situations.13,19,20 Tsukube and colleagues reported that magnesium cardioplegia and magnesium-supplemented potassium cardioplegia, which were used in the present study, both inhibit cytosolic Ca²⁺ accumulation in a manner similar to that seen with the combined use of ryanodine or nifedipine and potassium

Fig 3. Representative Western blot analysis of the microsomal fraction rich in sarcoplasmic reticulum vesicles from each group. Semiquantitative analysis using densitometry of the Western blots indicates that there were no significant differences in the expression levels of ryanodine receptor protein among the 3 groups.
cardioplegia during global ischemia. They concluded that the modulating action of magnesium-supplemented potassium cardioplegia on cytosolic Ca\(^{2+}\) accumulation was exerted through an inhibition of both the myocardial L-type Ca\(^{2+}\) channel and the SR RyR. The effects of magnesium on RyR have been examined by several investigators. Laver and coworkers reported that RyR possess 2 magnesium-sensitive gates (type I and II), which mediate magnesium inhibition via a reduction in the opening probability of RyR. In type I inhibition, magnesium competed with Ca\(^{2+}\) for high-affinity Ca\(^{2+}\)-activation sites thus preventing Ca\(^{2+}\) from opening the channel. Type II inhibition resulted from the binding of magnesium to low-affinity sites, which did not discriminate between Ca\(^{2+}\) and magnesium.

In the present study, we could not investigate the precise mechanisms underlying the preservation of RyR by cardioplegia. However, we speculate that the mechanisms might be as follows: (i) during global ischemia, RyR in an active conformation are downregulated by changes in the cytosolic environment (Ca\(^{2+}\) overload, change in pH, etc); and (ii) magnesium-supplemented potassium cardioplegia reduces intracellular Ca\(^{2+}\) overload in ways reported previously and prevents damage to the cytosolic microorgans, including the SR. As a result, the number of RyR in the functional state is preserved.

In conclusion, our main finding is that the number of RyR in the functional state is much better preserved during cardioplegia than during global ischemia, the degree of preservation being well correlated with the preservation of cardiac function. We conclude that cardioplegia protects the cardiac RyR, and postulate that this effect may result in the protection of SR function during ischemia–reperfusion.

References
2. Inui M, Saito A, Fleisher S: Isolation of the ryanodine receptor from cardiac sarcoplasmic reticulum and identity with the feet structures. J Biol Chem 1987; 262: 15637–15642
3. Meissner G, Henderson JS: Rapid calcium release from cardiac sarcoplasmic reticulum vesicles is dependent on Ca\(^{2+}\) and is modulated by Mg\(^{2+}\), adenine nucleotide, calmodulin. J Biol Chem 1987; 262: 3065–3073
23. Laver DR, Owen VJ, Junankar PR, Taske NL, Dulhunty AF, Lamb GD: Reduced inhibitory effect of Mg\(^{2+}\) on ryanodine receptor-Ca\(^{2+}\) release channels in malignant hyperthermia. Biophys J 1997; 73: 1913–1924
24. Eager KR, Dulhunty AF: Activation of the cardiac ryanodine receptor by sulfhydryl oxidation is modified by Mg\(^{2+}\) and ATP. J Membr Biol 1998; 163: 9–18