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Ruthenium Red-induced Changes in Myocardial Energetics During Normal Beat and Ventricular Fibrillation in Isolated Rat Hearts

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Abstract: Ventricular Fibrillation (VF) is known to alter myocardial energetics, but the mechanism of these changes has not been fully elucidated. We have recently demonstrated that the perfusion of isolated hearts with Ruthenium Red (RR), a blocker of the mitochondrial Ca^{2+} uniporter, converted pacing-induced sustained VF to Ventricular Tachycardia (VT). Here we investigated whether and if so how perfusion of the heart with RR during normal beating and during sustained VF changed the myocardial energetics. Phosphorus nuclear magnetic resonance spectroscopy and magnetization transfer (^{31}P NMR) were applied to isolated perfused rat hearts. Sustained VF was induced by burst pacing of the heart. In hearts beating normally, perfusion with RR (10 μM) significantly reduced Left Ventricular Developed Pressure (LVDP) and isovolumic contractile performance (expressed as the product of heart rate and LVDP). However, the amounts of Creatine Phosphate (CrP), ATP and their sum (CrP+ATP) did not significantly change as compared with those before the perfusion, suggesting that there existed a close balance between energy supply and demand during perfusion with RR. In contrast, during pacing-induced sustained VF, RR perfusion resulted in a decrease of inorganic phosphate (Pi) and in an increase of both CrP and ATP as compared with levels during sustained VF, although the RR-induced changes were not significant. In addition, RR perfusion increased the sum of high-energy phosphate compounds; that is, CrP+ATP, to almost the same level as that in hearts beating normally. This study demonstrated that the perfusion of isolated rat hearts with a blocker of the uptake of Ca^{2+} by mitochondria during sustained VF resulted in the close balance between myocardial energy supply and demand, suggesting that the balance was lost during VF and RR perfusion significantly ameliorated this imbalance.

Key words: ventricular fibrillation, normal beat, ^{31}P NMR, mitochondria, ruthenium red

INTRODUCTION

Ventricular Fibrillation (VF) and reentrant Ventricular Tachycardia (VT) are the major immediate causes of sudden cardiac death[1]. Whereas VT is considered a rapid but well organized process, VF has been described as turbulent cardiac electrical activity, resulting from the random and aperiodic propagation of multiple independent wavelets throughout the cardiac muscle[2]. Clinical studies have shown that VF is almost always preceded by VT of variable duration, from a few to many beats[3,4]. Also, in isolated rat hearts, a spontaneous transition from VT to VF is frequently observed during reperfusion after global ischemia[5]. We have recently demonstrated that the perfusion of isolated rat hearts with ruthenium red (RR) or Ru 360, blockers of Ca^{2+} uptake by mitochondria, resulted in a reversible conversion of pacing-induced sustained VF to VT, suggesting that changes in mitochondrial Ca^{2+} uptake were involved in the transition between VT and VF[6].

It is well known that oxygen consumption during VF increases compared with that when the heart is beating normally [7,8]. VF induces other abnormal events involving energy-related phosphate compounds in the myocardium such as increases in inorganic phosphate (Pi) and proton concentrations and decreases in creatine phosphate (CrP) and ATP [9,10]. Such changes in myocardial energetics are well recognized, but little is known about the cellular basis responsible for the metabolic changes during VF.

Ruthenium red (RR) is a hexavalent polysaccharide stain that inhibits the mitochondrial accumulation of Ca^{2+} both in isolated mitochondria *in vitro*[12] and in myocardium *in vivo*[1]. We have recently found that the perfusion of isolated hearts with RR results in the reversible conversion of a sustained VF to VT and the effect is antagonized by co-treatment with S(-)-Bay K8644, an activator of L-type Ca^{2+} channels, suggesting that the inactivation of L-type Ca^{2+} channels is responsible for the RR-induced effect on the macro-dynamics of hearts[6] . In addition, we have also

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demonstrated that perfusion with verapamil, a blocker of L-type calcium channels, during sustained VF resulted in the reversible conversion of VF to VT[13]. These findings have led to the suggestion that perfusion with RR during VF reduces myocardial energy expenditure for calcium transport processes via RR-induced decrease in the influx of Ca^{2+} through L-type Ca^{2+} channels. In contrast, the RR-induced inhibition of Ca^{2+} uptake by mitochondria is known to inhibit Ca^{2+}-dependent dehydrogenase activity in mitochondria, suggesting that perfusion with RR suppresses mitochondrial respiration resulting in the decreased production of ATP[14,15].

In this study, we have tried to elucidate whether and if so how the perfusion of isolated hearts with RR during normal beating and during sustained VF changed the myocardial energetics.

MATERIALS AND METHODS

The animal experiments conformed to the "Principles of laboratory animal care" (NIH publication No. 85-23, revised, 1996), as well as the "guide for the care and use of laboratory animals", Hokkaido University School of Medicine.

Preparation of Isolated Heart: A total of 25 male Wistar rats (10 weeks old) were anesthetized with diethylether and administered heparin at 400 μ kg^{-1} intravenously. The chest was opened, the aorta cannulated and the heart excised and immediately placed in ice-cold Krebs-Henseleit bicarbonate (KHB) buffer. The heart was then connected to the Langendorff apparatus and aortically perfused in a non-recirculating constant pressure mode. Hearts were perfused with a modified KHB solution containing the following (in mM): NaCl 100, KCl 5, $NaHCO_3$ 20, sodium acetate 20, $CaCl_2$ 2, glucose 10.

A water-filled elastic balloon was inserted into the left ventricle via the left atrium. The left ventricular pressure (LVP) was monitored by a pressure transducer (DT-XXED, Ohmeda, Madison, WI) connected to the balloon and was continuously monitored and recorded. The initial value of the End Diastolic Pressure (EDP) was set to 7-10 mmHg by adjusting the volume of the balloon. Because of the slight variation in the wet-weight of the isolated hearts from male Wistar rats (10 weeks old) used in this study, we did not normalize the amounts of inorganic phosphate (Pi), creatine phosphate (CrP) and ATP in the myocardium with the weight of each of the hearts.

Induction of Ventricular Tachyarrhythmias: Hearts were perfused for about 20 min with a modified KHB solution (equilibrium period). The hearts were then electrically stimulated through the right ventricle with an agar wick soaked in 2 M potassium chloride and encased in polyethylene tubing for the induction of Ventricular Fibrillation (VF). Burst pacing (pulse interval, 40-70 ms; pulse width, 1 ms; Intensity, 60-80 V) of the heart for 60 sec or more successfully induced a sustained VF in all the tested hearts (6 hearts).

Identification of Arrhythmia: A digital storage-type oscilloscope (VC-11, Nihon Kohden, Tokyo, Japan) and a thermal pen recorder were used in the identification and analysis of LVP waveforms and the diagnosis of arrhythmia. The EMG recordings of the left ventricular myocardium could not be performed during the measurement of ^{31}P NMR spectroscopy. Therefore, VF was defined as the development of a chaotic, irregular and non-pulsatile left ventricular pressure (LVP).

NMR Methodology: The ^{31}P NMR methods have been described previously[16] In summary, a Bruker MSL-200 spectrometer (Billerica, MA) was utilized for all experiments. The heart was lowered into a 25-mm diameter NMR tube and placed into the bore of a 4.7 Tesla superconducting magnet. ^{31}P NMR spectra were obtained at 81.01 MHz with 152 transients and a recycle time of 1.9 s. For each experiment, a fully relaxed spectrum, using a recycle time of 10 s, was acquired before the control period. The amounts of Pi, CrP and ATP in myocardium were calculated from the areas under their respective peaks using WIN-NMR software (Bruker, version 960901).

Drugs Used and Their Application: Ruthenium Red (RR) was obtained from Sigma (St. Louis, MO). The other chemicals were from Wako Chem. (Tokyo, Japan).

Statistics: The data are expressed as the mean±SD. Comparisons were performed using the one-way analysis of variance (ANOVA) followed by a paired t-test. A p value of less than 0.05 was considered statistically significant.

RESULTS

We first investigated whether and if so how the perfusion of isolated hearts with Ruthenium Red (RR) changed Left Ventricular Developed Pressure (LVDP), Heart Rate (HR) and the isovolumic contractile performance expressed as the product of heart rate and LVDP (Rate-pressure Product, RPP). The concentration of RR was set at 10 μM, since such a concentration consistently converted the pacing-induced sustained VF to VT in isolated rat hearts[5]. RR perfusion resulted in a significant but reversible decrease in the LVDP, but not in the heart rate (Fig. 1A, B, C1 and C2). Thus, the RPP

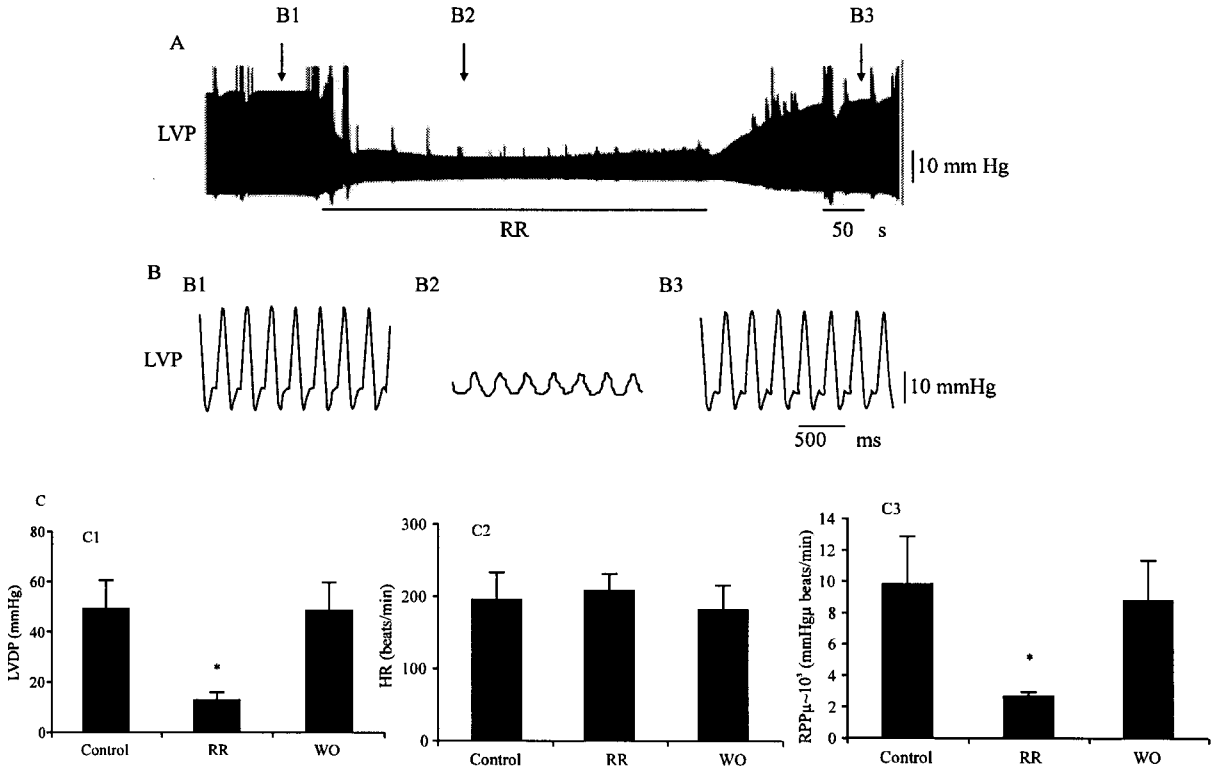

Fig. 1: Ruthenium red-induced changes in Left Ventricular Developed Pressure (LVDP), Heart Rate (HR) and Rate-pressure Product (RPP) during normal beating. Perfusion of isolated hearts with 10 μM Ruthenium Red (RR) significantly reduced the LVDP, but did not change the HR. Thus, the perfusion resulted in a significant decrease in the RPP, reflecting the isovolumic contractile performance in isolated hearts. Data are expressed as the Mean+SD (n=10). * $p<0.05$. LVP: Left Ventricular Pressure, LVDP, left ventricular developed pressure, HR: Heart Rate, RPP: Rate-pressure Product, WO, 5 min after the onset of washout

hearts with RR did not produce any marked changes in the amount of creatine phosphate (CrP) and ATP, or their sum (CrP+ATP). Inorganic phosphate (Pi) was hardly detected during the control period (before RR perfusion), during RR perfusion and during the washout of RR (Fig. 2A1-A3). These results suggested that perfusion with RR of normal beating hearts did not produce a noticeable change in the myocardial energy status, although it significantly reduced the RPP reflecting energy expenditure for rhythmic contraction as shown in Fig. 1C3. Thus, perfusion with RR possibly reduced the energy (ATP) production rate concurrently with the reduction of energy expenditure, resulting in the close balance between myocardial energy supply and demand.

We next investigated whether and if so how the perfusion with RR during rapid pacing-induced VF changed the myocardial energetics (Fig. 3). During VF, the amount of Pi was significantly increased compared with that during normal beating (control) as shown in Fig. 3B. In contrast, both ATP and CrP were markedly decreased (Fig. 3C and 3D). The perfusion of isolated hearts with RR resulted in a decrease in Pi and an increase in both ATP and CrP, although the changes were not significant. However, the sum of the myocardial high-energy phosphate compounds (ATP+CrP) increased to almost the same level as that during normal beating (control) caused by perfusion with RR during VF (Fig. 3E), suggesting that some balance between myocardial energy supply and demand was attained. After the washout of RR, Pi increased and ATP and CrP decreased and returned to the values before the onset of the perfusion; that is, those during VF. These results suggested that the balance between myocardial energy supply and demand was lost during VF and perfusion with RR significantly ameliorated this imbalance.

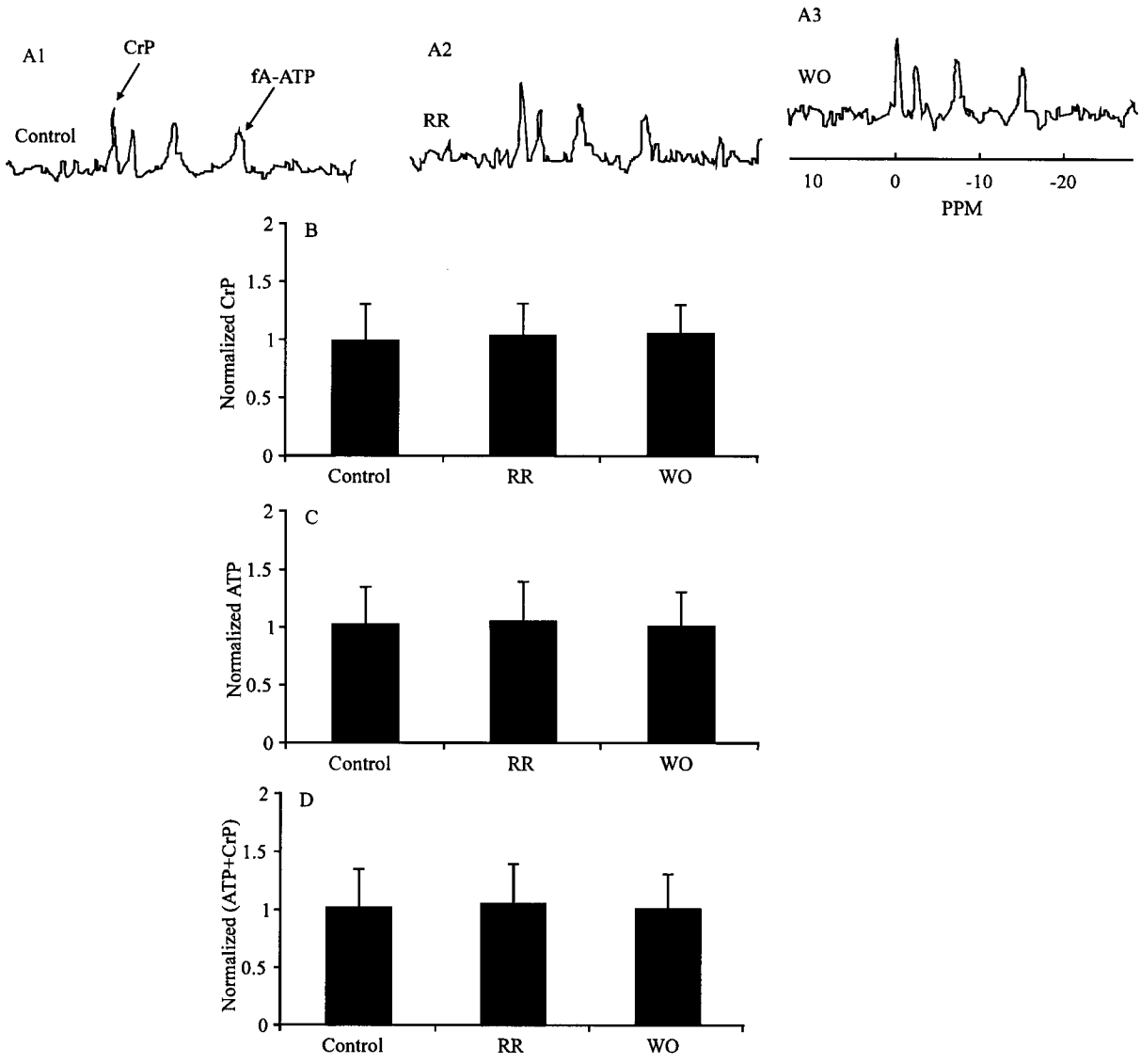

Fig. 2: Ruthenium red-induced changes in myocardial energetics in the normal beating hearts. Isolated rat hearts were perfused with 10 μM Ruthenium Red (RR) and a ^{31}P NMR spectral analysis was performed. The perfusion did not produce significant changes in the amounts of CrP, ATP and their sum (ATP+CrP). These results suggested that perfusion with RR during normal beating did not change myocardial energetics, although the perfusion significantly reduced the RPP reflecting energy expenditure for rhythmic contraction. The amounts of CrP, ATP and (ATP+CrP) are normalized using each of the averages before the onset of perfusion with RR (control). Data are expressed as the Mean+SD (n=10). CrP: creatine phosphate; WO, 5 min after the onset of washout

DISCUSSION

The present study demonstrated that perfusion of isolated rat hearts with a blocker of the uptake of Ca^{2+} by mitochondria during sustained VF resulted in a close balance between myocardial energy supply and demand, suggesting that the balance was lost during VF and the perfusion with RR significantly ameliorated this imbalance.

In this study, perfusion with Ruthenium Red (RR) during normal beating resulted in a significant reduction in Left Ventricular Developed Pressure (LVDP)

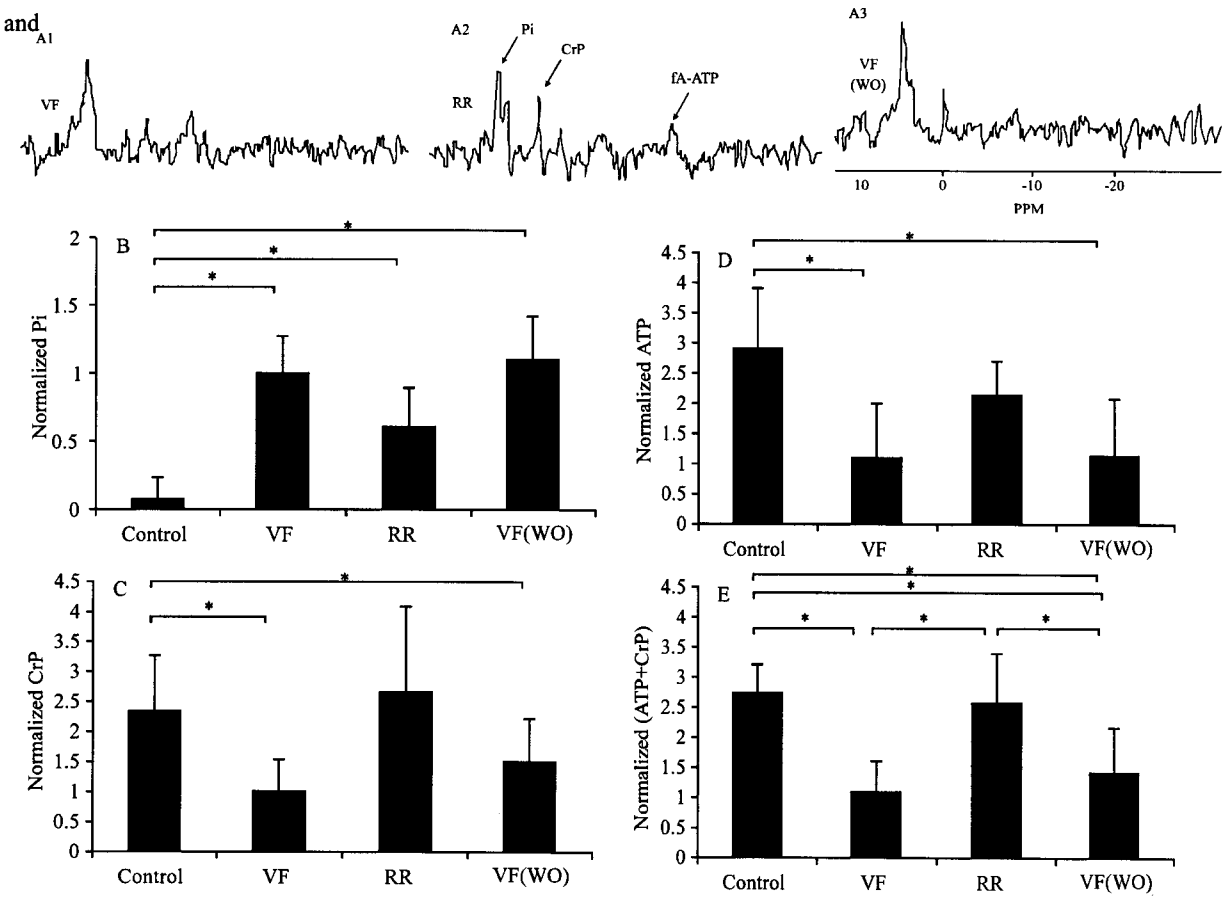

Fig. 3: Changes in the myocardial energetics of isolated hearts caused by perfusion with ruthenium red during rapid pacing-induced sustained ventricular fibrillation. During sustained ventricular fibrillation (VF), isolated rat hearts were perfused with 10 μM Ruthenium Red (RR) and a ^{31}P NMR spectral analysis was performed. During VF, the amount of Pi was significantly increased compared with that during normal beating (control), but that of both ATP and CrP was markedly decreased. Perfusion of isolated hearts with RR tended to decrease the amount of Pi, but increase that of both ATP and CrP, reversibly. The summation of ATP and CrP, myocardial high-energy phosphate compounds, increased to almost the same level as that during normal beating (control) caused by RR perfusion during VF. The amounts of Pi, CrP, ATP and (ATP+CrP) are normalized to each of the averages during sustained VF. Data are expressed as the mean+SD (n=6). * $p<0.05$. Pi: inorganic phosphate; VF: ventricular fibrillation; WO, 5 min after the onset of washout.

isovolumic contractile performance (expressed as the product of heart rate and LVDP) (Fig. 1). However, the amounts of Creatine Phosphate (CrP), ATP and their sum (CrP+ATP) did not change significantly as compared with those before the perfusion (Fig. 2), suggesting that there existed a close balance between myocardial energy supply and demand during perfusion with RR. We have previously suggested that the local Ca^{2+} rise in the micro-domains surrounding the mouth of L-type Ca^{2+} channels, SR and mitochondria caused by the RR-induced reduction of Ca^{2+} uptake by mitochondria would result in the reduced activity of sarco lemmal L-type Ca^{2+} channels [6]. In support of this idea, a recent study has revealed that the inhibition of mitochondrial Ca^{2+} uptake on treatment with RR or Ru 360 results in a reduction in the activity of L-type Ca^{2+} channels in rat hearts[17]. It is well known that the activity of L-type Ca^{2+} channels is negatively regulated by an increase in intracellular Ca^{2+} (Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels)[18]. These findings have led to the

notion that perfusion with RR reduced the amplitude of LVP and RPP reflecting isovolumic contractile performance in isolated hearts probably via an RR-induced decrease in the activity of L-type Ca^{2+} channels, resulting in a marked decrease in myocardial energy expenditure.

In this study, the perfusion of normal beating hearts with RR significantly reduced isovolumic contractile performance, but the amounts of CrP, ATP and (CrP+ATP) did not significantly change as compared with those before the perfusion (Fig. 2). These results suggested that perfusion with RR must concurrently reduce production of ATP in order to balance the myocardial energy supply and RR-induced reduction in energy demand. It has been known that intra-mitochondrial concentration of Ca^{2+} is a key regulator of mitochondrial ATP production via oxidative phosphorylation[19]. The transfer of Ca^{2+} across the inner membrane of mammalian mitochondria through the Ca^{2+} uniporter results in several steps of oxidative phosphorylation, including the activation of Ca^{2+}-dependent dehydrogenases[14,15] as well as production of ATP by F1,F0-ATPase[20]. These findings have led to the idea that RR-induced inhibition of Ca^{2+} uptake by mitochondria may reduce myocardial ATP production, balancing the RR-induced reduction in the myocardial energy demand.

The present study also demonstrated that perfusion with RR during pacing-induced sustained VF decreased the level of inorganic phosphate (Pi), but increased levels of ATP and CrP, resulting in a balance between myocardial energy supply and demand (Fig. 3). The metabolic deterioration that occurs during VF seems to be caused by increased cellular Ca^{2+} cycling across sarcolemma, sarcplasmic and mitochondrial membranes[21]. It is well known that oxygen consumption [22] and ATP usage increase dramatically during VF[9] as well as during atrial fibrillation[23,24].

It has been suggested that intracellular Ca^{2+} overload predisposes the myocardium to abnormal electrical activities promoting VF[25,26]. However, Merillat *et al.*[27] reported that an increase in cytosolic, global Ca^{2+} per se seems unnecessary for the initiation and maintenance of VF and that an increase of Ca^{2+} influx through L-type Ca^{2+} channels (slow Ca^{2+} channels) is essential. In fact, previous studies including a report by us[13,28,29] have revealed that the perfusion of isolated hearts with blockers of L-type Ca^{2+} channel activities such as verapamil during sustained VF results in the reversible conversion of VF to VT. These results suggest that an increase of Ca^{2+} influx through L-type Ca^{2+} channels occurs during VF. We have recently demonstrated the possibility that inhibition of mitochondrial Ca^{2+} uptake by RR perfusion during sustained VF results in a reversible conversion from VF to VT via Ca^{2+}-dependent inactivation of L-type Ca^{2+} channel activities[6]. All these findings have led to the suggestion that perfusion with RR during pacing-induced sustained VF drastically decreased myocardial energy demand via RR-induced inhibition of the activity of L-type Ca^{2+} channels, resulting in a balance between myocardial energy supply and demand.

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