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Attenuation of mitochondrial, but not cytosolic, Ca^{2+} overload reduces myocardial injury induced by ischemia and reperfusion[1]

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Key words

ruthenium red; BAPTA-AM; infarct size; electrically-induced Ca^{2+} transient; isolated perfused rat heart; cardiac myocytes

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Abstract

Aim: Attenuation of mitochondrial Ca^{2+} ($[Ca^{2+}]_m$), but not cytosolic Ca^{2+} ($[Ca^{2+}]_c$), overload improves contractile recovery. We hypothesized that attenuation of $[Ca^{2+}]_m$, but not $[Ca^{2+}]_c$, overload confers cardioprotection against ischemia/reperfusion-induced injury. **Methods:** Infarct size from isolated perfused rat heart, cell viability, and electrically-induced Ca^{2+} transient in isolated rat ventricular myocytes were measured. We determined the effects of BAPTA-AM, a Ca^{2+} chelator, at concentrations that abolish the overload of both $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$, and ruthenium red, an inhibitor of mitochondrial uniporter of Ca^{2+} transport, at concentrations that abolish the overload of $[Ca^{2+}]_m$, but not $[Ca^{2+}]_c$, on cardiac injury induced by ischemia/reperfusion. **Results:** Attenuation of both $[Ca^{2+}]_m$ and $[Ca^{2+}]_c$ by BAPTA-AM, and attenuation of $[Ca^{2+}]_m$, but not $[Ca^{2+}]_c$, overload by ruthenium red, reduced the cardiac injury observations, indicating the importance of $[Ca^{2+}]_m$ in cardioprotection and contractile recovery in response to ischemia/reperfusion. **Conclusion:** The study has provided unequivocal evidence using a cause-effect approach that attenuation of $[Ca^{2+}]_m$, but not $[Ca^{2+}]_c$, overload is responsible for cardioprotection against ischemia/reperfusion-induced injury. We also confirmed the previous observation that attenuation of $[Ca^{2+}]_m$, but not $[Ca^{2+}]_c$, by ruthenium red improves contractile recovery following ischemia/reperfusion.

Introduction

Ischemia and reperfusion (I/R) induces myocardial injury. Cytosolic Ca^{2+} ($[Ca^{2+}]_c$) is believed to be a precipitating factor. There is increasing evidence, however, showing that mitochondria, which has a huge capacity to accumulate[1–3] and buffer Ca^{2+} under condition of high $[Ca^{2+}]_c$ loading[4], plays a critical role in the maintenance of cellular Ca^{2+} homeostasis in health and disease[5]. When the heart is subjected to hypoxia or ischemia, $[Ca^{2+}]_c$ increases. When $[Ca^{2+}]_c$ increases, mitochondria take up Ca^{2+} from cytosol. Accumulation of Ca^{2+} in mitochondria ($[Ca^{2+}]_m$) is believed to be the cause of irreversible ischemic injury[6], therefore attenuation of $[Ca^{2+}]_m$ should be cardioprotective. In support of this, a nitric oxide (NO) donor has been shown to protect the heart against I/R injury accompanied by a reduction in $[Ca^{2+}]_m$, suggested by the authors to be a likely mechanism for NO-induced protection[7]. It has also been reported that opening of the ATP-sensitive potassium channel, which confers cardioprotection induced by ischemic insults, is accompanied by attenuation of mitochondrial Ca^{2+} overload and reduction in mitochondrial membrane potential[8]. The observation, which was based on a study of correlative nature, also supports that cardioprotection might be the consequence of attenuation of Ca^{2+} overload and/or reduction in mitochondrial membrane potential. In fact it was shown that in the heart subjected to ischemia, recovery in contractile function during reperfusion is improved after ruthenium

red is given, which significantly attenuated the $[Ca^{2+}]_m$, but not $[Ca^{2+}]_c$, overload[9]. This evidence is based on a cause–effect study indicating that $[Ca^{2+}]_m$ plays a crucial role in cardiac injury and recovery after I/R. Unfortunately, myocardial injury was not measured in the study.

In the present study we determined myocardial injury induced by I/R *in vivo* and *in vitro* with manipulations that attenuated the overload of either $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$, or $[Ca^{2+}]_m$ alone. We also studied the contractile function *in vivo*. BAPTA-AM, a Ca^{2+} chelator, was used to attenuate the overload of both $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$, whereas ruthenium red, an inhibitor of the uniporter of Ca^{2+} transport across the mitochondrial membrane, was used to attenuate the overload of $[Ca^{2+}]_m$. Results are unequivocal evidence that $[Ca^{2+}]_m$, but not $[Ca^{2+}]_c$, is the immediate cause of myocardial injury induced by myocardial I/R.

Materials and methods

Isolated perfused heart preparation The protocols of this study were approved by the Committee on the Use of Experimental Animals for Teaching and Research, The University of Hong Kong (Hong Kong SAR, China). Male Sprague-Dawley rats of 250–300 g body weight were anesthetized with sodium pentobarbitone (60 mg/kg, ip) and given heparin (200 IU, iv). Hearts were excised rapidly and placed in ice-cold Krebs-Henseleit (K-H) perfusion buffer before being mounted on a Langendorff apparatus. Hearts were perfused at 37 °C with K-H buffer at a constant pressure (100 cm H_2O) and equilibrated with 95% O_2/5% CO_2. The buffer contained (mmol/L): NaCl 118.0, KCl 4.7, $CaCl_2$ 1.25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 11.0. For hearts subjected to regional ischemia, a silk suture was placed around the left coronary artery to form a snare. The coronary artery was occluded by pulling the snare to produce ischemia. Reperfusion was achieved by releasing the occlusion. In the present study, the isolated heart was subjected ischemia for 30 min followed by reperfusion for 120 min, known to induce myocardial injury. A balloon was inserted through the left atrium into the left ventricle and the left ventricular end diastolic pressure (LVEDP) was adjusted to between 4 and 8 mmHg. Cardiac parameters, namely, heart rate (HR), left ventricular developed pressure (LVDP) and velocity of contraction and relaxation ($\pm dP/dt_{max}$), were monitored continuously. Coronary flow, expressed in mL/min, was measured by timed collection of effluent at regular intervals, using a calibrated tube.

Measurement of the area of risk For determination of infarct size, the coronary artery was re-occluded at the end of reperfusion and a solution with 2.5% Evans blue was perfused to determine the area of risk. Hearts were then frozen and cut into slices, which were then incubated in a sodium phosphate buffer containing 1% (*w/v*) 2,3,5-triphenyl-tetrazolium chloride for 15 min to visualize the unstained infarcted region. Infarct and risk zone areas were determined by planimetry with the software Image/J from NIH. The area at risk was expressed as a percentage of the left ventricle. The infarct size was expressed as a percentage of the risk zone. The risk zones in different groups were similar to each other (Figure 1, 3).

Preparation of isolated ventricular myocytes Single ventricular myocytes were prepared from the hearts of male Sprague-Dawley rats by enzymatic dissociation[10]. The heart was perfused using a Langendorff apparatus with a 100% oxygenated, non-recirculating Ca^{2+}-free Tyrode's solution. The perfusion solution was switched to a 100% oxygenated recirculated, low Ca^{2+} (50 μmol/L) Tyrode's solution containing 0.03% collagenase and 1% bovine serum albumin (BSA) for 10 min. The ventricles were cut, minced, and gently triturated with a pipette in the low Ca^{2+} Tyrode's solution containing BSA at 37 °C for 10 min. The cells were filtered through 200 mm nylon mesh and re-suspended in the Tyrode's solution, in which the Ca^{2+} concentration was gradually increased to 1.25 mmol/L in 40 min. Only rod-shaped cells with clear cross-striations were used. For ischemic insults, myocytes were incubated for 10 min with a solution containing 10 mmol/L 2-deoxy-*D*-glucose and 10 mmol/L sodium dithionite ($Na_2S_2O_4$), that induce metabolic inhibition and anoxia (MI/A)[11,12], two consequences of ischemia. This was followed by perfusion with normal K-H solution for 10 min.

Cell viability Trypan blue exclusion was used as an index of the viability of the myocytes[13,14]. After cells were incubated with 0.4% trypan blue dye for 3 min, they were counted in a hemocytometer chamber under a light microscope. Dead cells are not able to exclude trypan blue and thus appear blue. The cell morphology was determined by microscopic examination[12]. Only rod-shaped (length: width, >3:1) cells were used for data collection.

Intracellular Ca^{2+} recording Intracellular Ca^{2+} and its transient were determined by a spectrofluorometric method using the sensitive dye Fura-2 as Ca^{2+} indicator. Loading of cells with Fura-2/AM was carried out as described previously[10]. After stabilization, isolated myocytes were incubated with 1 mmol/L Fura-2/AM at room temperature for 30 min. The loaded cells were washed 3 times with fresh K-H buffer solution containing 1% BSA to wash out the unincorporated Fura-2/AM. The myocytes were kept at room tem-

perature for approximately 30 min to allow complete hydrolysis of acetoxymethyl ester groups and generate Ca^{2+}-sensitive Fura-2 free anion. The Ca^{2+}-dependent signal of Fura-2 was obtained by illuminating at 340 and 380 nm and recording the emitted light at 510 nm. The fluorescence ratio (F_{340}/F_{380}) of Fura-2/AM loaded myocytes represents resting $[Ca^{2+}]_c$ level.

Measurement of $[Ca^{2+}]_m$ The freshly isolated ventricular myocytes were incubated with 10 mmol/L Rhod-2/AM[15] in normal Tyrode's solution for 2 h at 37 °C. Myocytes were then washed with 1.25 mmol/L Ca^{2+} Tyrode's solution to get rid of extracellular Rhod-2/AM. The myocytes were kept at room temperature for approximately 30 min to allow complete hydrolysis of acetoxymethyl ester groups of mitochondrial dye. Confocal images were acquired using an Olympus 1X71 inverted confocal microscope (Olympus, Japan) with a 40× water immersion objective. Rhod-2 was excited by a 543 nm helium-neon laser and emission fluorescence was captured at >560 nm. Images were acquired in the frame scan mode with 512/512 pixels once every min before and during MI/A and reperfusion (MI/AR). The background fluorescence was estimated by taking an image in the absence of myocytes. The Rhod-2 fluorescence was determined by taking an image in the presence of myocytes. The background fluorescence was then subtracted from the Rhod-2 fluorescence images. The relative Rhod-2 fluorescence was quantified as F/F_0, where F was the fluorescence intensity of myocytes, and F_0 was the fluorescence intensity before MI/A.

Experimental protocols Rats were anesthetized with pentobarbital, then injected with BAPTA-AM at 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg or 2.5 mg/kg, iv, ruthenium red (10 mg/kg, iv) or dimethylsulfoxide (Me_2SO) as the solvent control through cannulated femoral veins. After one h, the time it takes for the drug to take effect[16], the rats were killed and the hearts removed.

Ventricular myocytes were subjected to continuous perfusion (the vehicle control) MI/A followed by reperfusion with or without drug treatment. Two drugs, namely BAPTA-AM (1 μmol/L, 2.5 μmol/L, or 25 μmol/L) and ruthenium red (50 mmol/L), were given during ischaemia for 10 min, then during reperfusion for another 10 min.

Drugs and chemicals Eagle's mininal essential medium, type I collagenase, BSA, 2-deoxy-*D*-glucose, Fura-2/AM, BAPTA-AM and trypan blue were purchased from Sigma Chemical (St Louis, MO, USA). Ruthenium red was purchased from TOCRIS. Sodium dithionite was purchased from Merck and Tocris Cookson. Rhod-2/AM was purchased from Molecular Probes. All chemicals were dissolved in distilled water or Krebs solution, except Fura-2/AM, Rhod-2/AM, calcein/AM and BAPTA-AM, which were dissolved in Me_2SO at a final Me_2SO concentration of <0.1%, which itself had no effect[17]. The concentration of BAPTA-AM was chosen according to previous studies[16,18]. Ruthenium red (10 mg/kg, iv) was injected according to a previous study of Malinowska *et al*[19]. The present study showed that ruthenium red at this concentration did not affect the contractile function measured in isolated perfused rat heart, which is in agreement with the observation of the previous study[19]. Ruthenium red 50 μmol/L was chosen for experiments in the isolated ventricular myocytes preparation based on the concentration used in systemic administration, distributed in the extracellular fluid compartment volume of approximately 20% of the body weight. In our preliminary experiments we found that at this concentration ruthenium red had no significant effect on the electrically-induced $[Ca^{2+}]_i$ transient.

Statistical analysis Values are presented as means±SEM. Statistical comparisons were made using one-way ANOVA and the Newman-Keuls test. $P<0.05$ was considered statistically significant.

Results

Effects of BAPTA-AM on infarct size in isolated rat hearts subjected myocardial I/R The infarct sizes of the isolated hearts after 30 min ischaemia and 2 h reperfusion after injection of 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg and 2.5 mg/kg BAPTA-AM were 35.92%±1.57%, 31.83%±1.11%, 30.95%±1.88% and 31.09%±1.97%, respectively (Figure 1). The infarct sizes of the groups receiving 1 mg/kg, 1.5 mg/kg and 2.5 mg/kg BAPTA-AM were significantly smaller than that of the control with injection of Me_2SO only (37.6%±2.34 %). The observation indicates that BAPTA-AM reduces cardiac injury when given *in vivo*.

Effects of MI/AR on percentage of non-blue cells in ventricular myocytes treated with BAPTA-AM The percentage of non-blue (live) cells was 44.55%±1.41% at the end of reperfusion, significantly lower than that of the vehicle control (61.69%±2.55 %) (Figure 2), indicating that MI/AR increases cell death.

In the groups treated with 1, 2.5 and 25 μmol/L BAPTA-AM, the percentages of non-blue cells at the end of reperfusion were 45.6%±2.39%, 48.4%±1.14%, and 53.04%± 2.05 %, respectively (Figure 2). The values were significantly higher than in the group subjected to MI/AR only (44.55%±1.41%). The results indicate that BAPTA-AM at 2.5 and 25 μmol/L reduces cell death.

Effects of myocardial I/R on infarct size In the hearts receiving 50 μmol/L ruthenium red, the infarct size was sig-

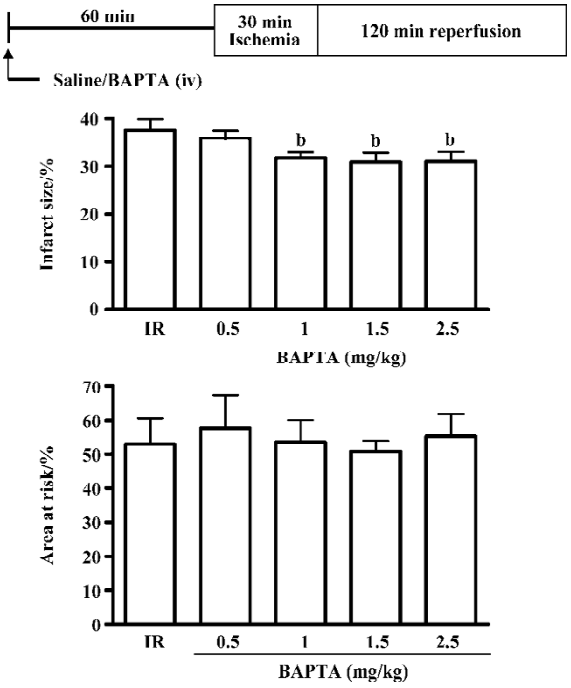

Figure 1. Effects of ischemia (30 min) and reperfusion (2 h) on myocardial infarct in the isolated perfused rat heart treated with BAPTA-AM. Infarct size was determined at the end of reperfusion. The experimental protocol is shown in the upper panel. BAPTA-AM (0.5, 1, 1.5, and 2.5 mg/kg) was injected 1 h before ischemia. Mean±SEM. n=10. ^bP<0.05 *vs* control.

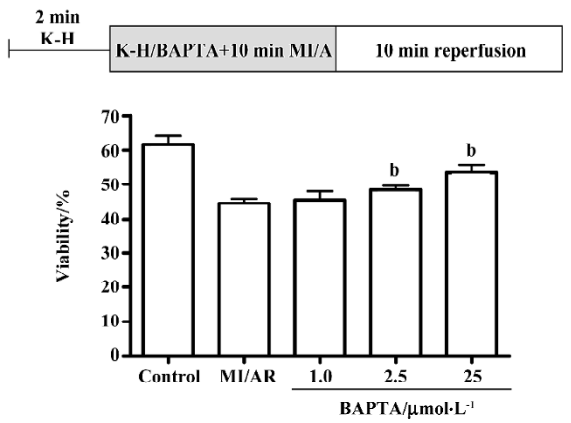

Figure 2. Effects of BAPTA-AM on trypan blue exclusion of rat ventricular myocytes subjected to MI/AR. The experimental protocol is shown in the upper panel. BAPTA-AM (1–25 μmol/L) was injected during 10 min MI/A. Mean±SEM. n=10. ^bP<0.05 *vs* control.

nificantly reduced compared with the vehicle control group (Figure 3), indicating ruthenium red attenuates injury induced by myocardial ischemia and reperfusion.

Effects of MI/AR on percentage of viable cells in ventricular myocytes treated with ruthenium red In the

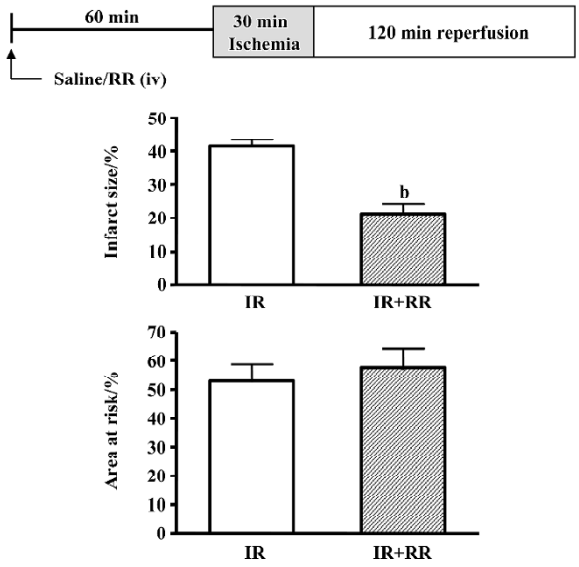

Figure 3. Effects of ischemia (30 min) and reperfusion (2 h) on myocardial infarct in the isolated perfused rat heart treated with ruthenium red (RR). The experimental protocol is shown in the upper panel. Ruthenium red (10 mg/kg) was injected 1 h before ischemia. Values are presented as mean±SEM. n=10 for each group. ^bP<0.05 *vs* I/R.

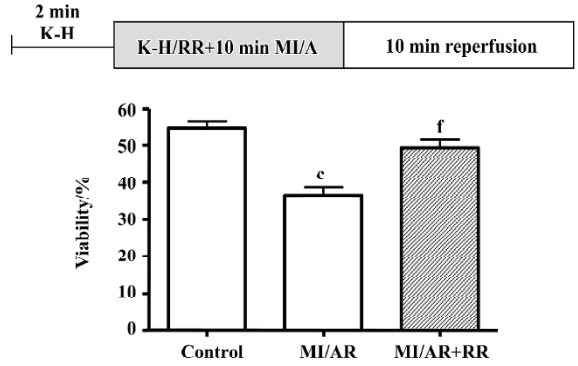

Figure 4. Effects of ruthenium red (RR, 50 μmol/L) on trypan blue exclusion of rat ventricular myocytes subjected to MI/AR. The experimental protocol is shown in the upper panel. RR was injected during 10 min MI/A. Mean±SEM. n=10 in each group. ^cP<0.01 *vs* control. ^fP<0.01 *vs* MI/AR group.

myocytes treated with ruthenium red at 50 μmol/L, the percentage of non-blue cells was significantly greater than that of the group subjected to MI/AR only (Figure 4), indicating a protective effect of ruthenium red against ischemic insult.

Effects of MI/AR on ventricular function and coronary flow Ischemic insults resulted in marked decreases in LVDP, the rate-pressure product (LVDP×HR), $\pm dP/dt_{max}$ and coronary flow and a significant increase in LVEDP during reperfusion in the isolated perfused rat heart (Table 1). Prior

Table 1. Hemodynamic parameters and coronary flow (CF) in the isolated perfused rat heart subjected to 30 min ischemia then 120 min reperfusion. Animals were injected with Me_2SO (control; n=12), BAPTA-AM (2.5 mg/kg; n=8) or ruthenium red (RR; 50 mmol/L; n=8). Values are expressed as mean±SEM. $^bP<0.05$ vs control (Me_2SO) group.

Variable		Baseline	Reperfusion		
			10 min	60 min	120 min
LVDP (mmHg)	Me_2SO	112.4±3.4	78.8±2.9	62.5±3.3	49.0±2.0
	BAPTA-AM	102.1±6.2	96.8±6.7^b	91.1±7.5^b	74.0±7.8^b
	RR	103.1±3.9	96.1±4.7^b	94.9±6.1^b	83.4±7.2^b
LVEDP (mmHg)	Me_2SO	8.1±1.4	17.2±2.0	19.8±1.3	24.8±1.4
	BAPTA-AM	7.5±0.4	15.5±2.7	11.8±1.5^b	13.8±4.4^b
	RR	9.0±0.9	10.9±2.7^b	8.5±2.3^b	11.2±3.1^b
$+dP/dt_{max}$ (mmHg/s)	Me_2SO	2635±47	1604±54	1495±39	1259±43
	BAPTA-AM	2510±76	2545±73*	2314±97^b	1749±95^b
	RR	2423±83	2899±61^b	3049±71^b	2868±85^b
$-dP/dt_{max}$ (mmHg/s)	Me_2SO	1758±56	1359±96	1267±53	918±78
	BAPTA-AM	1915±54	1827±123^b	1307±48	1360±56^b
	RR	1982±49	2297±95^b	2489±65^b	2322±79^b
LVDP×HR	Me_2SO	29302±961	20511±1873	16712±1126	11510±843
	BAPTA-AM	29020±964	24502±2083^b	20030±987^b	15150±423^b
	RR	27747±891	22604±2703	24048±943^b	19306±711^b
HR (bpm)	Me_2SO	273.6±5.6	260.3±14.5	267.4±12.4	234.9±10.9
	BAPTA-AM	284.4±8.9	253.8±11.2	216.2±10.6	203.4±15.1
	RR	269.2±6.8	276.7±5.8^b	254.5±16^b	233.2±9.1
CF (mL/min)	Me_2SO	8.6±0.6	7.5±0.3	4.1±0.3	3.6±0.2
	BAPTA-AM	8.4±0.7	7.1±0.3	6.3±0.8^b	4.9±0.4^b
	RR	8.7±0.9	7.9±0.7	7.3±0.3^b	5.0±0.6^b

injection of either 2.5 mg/kg BAPTA-AM or 50 μmol/L ruthenium red attenuated the effects of ischemic insults on all these parameters (Table 1). Ischemic insult also reduced the heart rate slightly at the end of reperfusion. Neither BAPTA-AM nor ruthenium red attenuated the effect of ischemic insult. Interestingly, in the BAPTA-AM treated group the heart rate was further reduced after 60 min reperfusion (Table 1).

Effects of MI/AR on fluorescence of F_{340}/F_{380} in ventricular myocytes treated with BAPTA-AM or ruthenium red During MI/A, the fluorescence ratio of F_{340}/F_{380}, which represents the $[Ca^{2+}]_c$ level, was gradually increased. Immediately after reperfusion, the ratio dropped, but was still higher than the original basal level. The fluorescence ratio during MI/AR was significantly higher than that of the vehicle control (Figure 5). This result indicates that $[Ca^{2+}]_c$ was increased during reperfusion.

When myocytes were treated with BAPTA-AM at 1 mmol/L, 2.5 μmol/L and 25 μmol/L, the F_{340}/F_{380} ratios at the end of MI/A were 1.04±0.008, 1.02±0.007 and 1±0.007, respectively, significantly lower than the group subjected to MI/AR only (1.07±0.006). The fluorescence ratios after 2 min of reperfusion were 1.01±0.05, 1.01±0.037 and 0.99±0.019, respectively, also significantly lower than that of the group subjected to MI/AR only (1.03±0.008) (Figure 5). This shows that BAPTA-AM attenuates $[Ca^{2+}]_c$ overload induced by MI/AR.

However, in myocytes treated with ruthenium red at 50 μmol/L, the fluorescence ratios at the end of MI/A and after 2 min of reperfusion were the same as those in the group subjected to MI/AR (Figure 5), indicating that ruthenium red does not affect $[Ca^{2+}]_c$.

Effects of MI/AR on F/F_0 of Rhod-2 The F/F_0 of Rhod-2, which represents the $[Ca^{2+}]_m$ level, did not change during MI/A, but increased significantly during reperfusion (Figure 6).

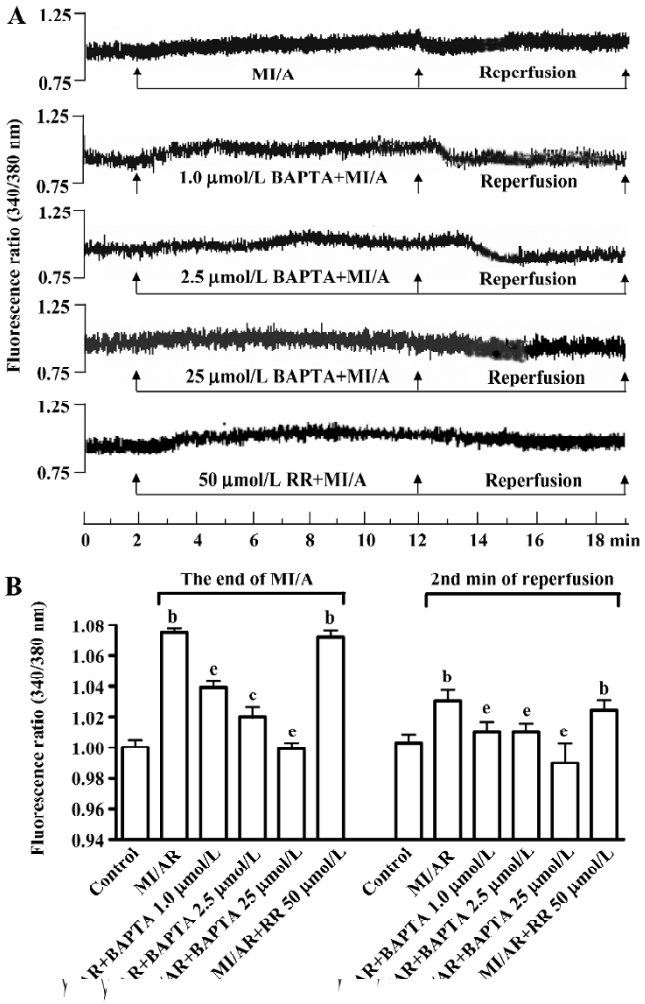

Figure 5. Effects of BAPTA-AM (1–25 μmol/L) on F_{340}/F_{380} of rat ventricular myocytes subjected to MI/AR. (a) Representative tracings; (b) group results showing florescence ratio at the end of MI/A and after 2 min of reperfusion. Mean±SEM. n=10 in each group. $^bP<0.05$ *vs* control. $^eP<0.05$ *vs* MI/AR group.

As the effect of BAPTA-AM was the greatest at 25 μmol/L on $[Ca^{2+}]_i$, we determined the F/F_0 in myocytes treated with 25 μmol/L BAPTA-AM. From 6 min of MI/A to the end of reperfusion, the F/F_0 was significantly lower than that of the group subjected to MI/AR (Figure 6). Interestingly, it was even lower than that of the control group without any treatment (Figure 6). This indicates that BAPTA-AM abolishes $[Ca^{2+}]_m$ overload induced by MI/AR. Ruthenium red at 50 μmol/L also attenuated the F/F_0 at the end of MI/A and after 2 min of reperfusion (Figure 6).

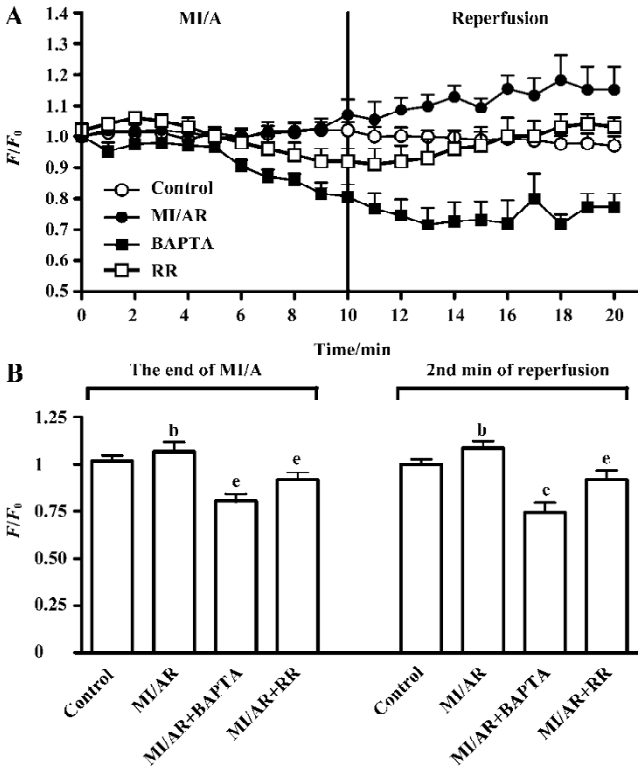

Figure 6. Effects of BAPTA-AM (1–25 μmol/L) on $[Ca^{2+}]_m$ of rat ventricular myocytes subjected to MI/AR. Mean±SEM. n=10 in each group. $^bP<0.05$ *vs* control. $^eP<0.05$ *vs* MI/AR group.

Discussion

A previous study has shown that treatment with ruthenium red, that attenuates $[Ca^{2+}]_m$ overload, improves the recovery of contractile functions, indicating that attenuated $[Ca^{2+}]_m$, but not $[Ca^{2+}]_c$, is responsible for recovery of contractile functions *in vivo*[19]. In agreement with this result, we also found in the present study that attenuation of $[Ca^{2+}]_m$, but not $[Ca^{2+}]_c$, overload with 50 μmol/L ruthenium red improved the recovery of contractile functions impaired by I/R in the isolated perfused rat heart. More importantly, we observed that ruthenium red reduced myocardial injury induced by ischemic insult and reperfusion in both the whole heart and isolated ventricular myocyte preparations. The observations are based on a cause-effect study that showed $[Ca^{2+}]_m$ is crucial in cardiac injury/protection[20]. The observations are also in agreement with the finding that opening of the mitochondrial ATP-sensitive potassium channel confers cardioprotection and reduces mitochondrial Ca^{2+} overload during I/R, suggesting that a reduction in mitochondrial Ca^{2+} overload might be responsible for cardioprotection[21]. They are also in agreement with a recent finding that $[Ca^{2+}]_m$ accumulation is crucial in burn injury-induced myocardial inflammation and function[22] .

In the present study we found that BAPTA-AM reduced

injury induced by ischemic insult and reperfusion. This is in agreement with the previous finding that BAPTA-AM reduces programmed myocyte cell death induced by angiotensin II[23]. We also found that BAPTA-AM attenuated the overload of both $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$. Ruthenium red overload, as shown in the present study, reduced injury induced by I/R, therefore the cardioprotective effect of the Ca^{2+} chelator must be due to attenuation of $[Ca^{2+}]_m$, but not $[Ca^{2+}]_c$, overload. So $[Ca^{2+}]_c$ overload induced by I/R leads to $[Ca^{2+}]_m$ overload, which precipitates myocardial injury.

A previous study has shown that an NO donor protects the cardiomyocytes of neonatal rats against I/R-induced injury, which is accompanied by attenuation of $[Ca^{2+}]_m$[7]. The authors suggested that the attenuation of $[Ca^{2+}]_m$ was a likely cause of NO-induced protection. Our study provided strong support to the suggestion.

It has also been suggested that hypercontracture, a predominant feature of reperfusion injury, is a cause of I/R-induced injury as control of hypercontracture reduces the extent of injury. One of the causes of hypercontracture might be high $[Ca^{2+}]_c$ when energy recovery is rapid[7]. This does not seem to be in total agreement with the finding that attenuation of $[Ca^{2+}]_m$, but not $[Ca^{2+}]_c$, is responsible for cardioprotection and improved contractile recovery. Further study is needed.

Ruthenium red at a concentration range up to 100 μmol/L has been shown to inhibit the efflux of Ca^{2+} by way of the ryanodine receptor of the sarcoplasmic reticulum of both skeletal and cardiac muscles[18]. This might reduce the $[Ca^{2+}]_c$, which might in turn reduce $[Ca^{2+}]_m$. In the present study we observed that ruthenium red at 50 mmol/L did not affect $[Ca^{2+}]_c$ at the end of MI/A and after 2 min of reperfusion, indicating that the inhibitory effect of ruthenium red on Ca^{2+} efflux by way of the ryanodine receptor did not affect the effects of MI/A or reperfusion on $[Ca^{2+}]_c$. The inhibitory effect of Ca^{2+} efflux might also increase the Ca^{2+} content in the sarcoplasmic reticulum. There is, however, no evidence of the direct influence of Ca^{2+} content in the sarcoplasmic reticulum on cardiac function.

In the present study we observed that abolition of both mitochondrial and cytosolic Ca^{2+} overload with BAPTA-AM resulted in small reductions in myocardial infarct size than abolition of the mitochondrial and cytosolic Ca^{2+} overload with ruthenium red. This is not due to the degree of attenuation in Ca^{2+} overload, as the mitochondrial Ca^{2+} level was lower after treatment with BAPTA-AM than with ruthenium red. One possible explanation is that ruthenium red might itself have cardioprotective action in addition to its inhibitory effect on Ca^{2+} transport across the mitochondrial membrane. Further study is warranted.

In conclusion, the present study has provided unequivocal evidence based on a cause-effect study that, in addition to improved recovery of contractile function, attenuation of $[Ca^{2+}]_m$, but not $[Ca^{2+}]_c$, is responsible for reduced injury induced by I/R.

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