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Diastolic Ca^{2+} overload caused by Na^+/Ca^{2+} exchanger during the first minutes of reperfusion results in continued myocardial stunning

Geng-Ze Wei [a], Jing-Jun Zhou [a], Bo Wang [a], Feng Wu [b], Hui Bi [a], Yue-Min Wang [a], Ding-Hua Yi [c], Shi-Qiang Yu [c,*], Jian-Ming Pei [a,b,*]

[a] *Department of Physiology, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, PR China*
[b] *Department of Cardiology, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, PR China*
[c] *Department of Cardiac Surgery, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, PR China*

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Abstract

The pathogenesis of myocardial stunning caused by brief ischemia and reperfusion remains unclear. The aim of the present study was to investigate the underlying mechanism of myocardial stunning. An isolated cell model of myocardial stunning was firstly established in isolated rat ventricular myocytes exposed to 8 min of simulated ischemia and 30 min of reperfusion, the cardiomyocyte contractile function was used to evaluate myocardial stunning. A diastolic Ca^{2+} overload without significant changes in systolic Ca^{2+} and the amplitude of Ca^{2+} transient during the first 10 min of reperfusion played an important role in the occurrence of myocardial stunning. Decreasing Ca^{2+} entry into myocardial cells with low Ca^{2+} reperfusion was a very efficient way to prevent myocardial stunning. Diastolic Ca^{2+} overload was closely related to the reverse mode of Na^+/Ca^{2+} exchanger (NCX) rather than L-type Ca^{2+} channel. The activity of the reverse mode of NCX was found significantly higher at the initial time of reperfusion, and KB-R7943, a selective inhibitor of the reverse mode of NCX, administered at first 10 min of reperfusion rather than at the time of ischemia significantly attenuated myocardial stunning. In addition, NCX inhibition also attenuated the Ca^{2+} oscillation and cardiac dysfunction when field stimulus was stopped at first 10 min of reperfusion. These data suggest that one of the important mechanisms of triggering myocardial stunning is diastolic Ca^{2+} overload caused by activation of the reverse mode of NCX of cardiomyocytes during the initial period of reperfusion following brief ischemia.

Keywords: Diastolic Ca^{2+} overload; Na^+/Ca^{2+} exchanger; Myocardial stunning

1. Introduction

Myocardial stunning is a prolonged, reversible contractile dysfunction after reperfusion following brief ischemia (Braunwald and Kloner, 1982). The phenomenon becomes very common in clinic after widespread use of interventional recanalization and other methods for treatments of acute myocardiac ischemia (Bolli, 1992). With regard to pathophysiology of stunned myocardium, some studies have shown that stunned cardiomyocytes appear to be the changes in cellular important proteins, which contain sarcoplasmic reticulum (SR) Ca^{2+}-ATPase, ryanodine receptor, and troponin I (Gao et al., 1997; Gao et al., 1996; Kim et al., 2001; Krause et al., 1989; Valdivia et al., 1997). There are also changes in action potential and a decrease in L-type Ca^{2+} current density (Kim et al., 2001). However, it is more important to elucidate the pathogenesis in order to efficiently prevent myocardial stunning in clinic. Ca^{2+} overload is presumed to be one of the major mechanisms of triggering myocardial stunning because it has been found that ischemia and early reperfusion lead to Ca^{2+} overload by measurement of intracellular Ca^{2+} concentration (Carrozza et al., 1992; Kusuoka et al., 1987; Louch et al., 2002, 2005). Moreover, it is presumed that Ca^{2+} overload is mediated by the reverse mode of Na^+/Ca^{2+} exchanger (NCX), and acidosis during reperfusion inhibits the reverse mode of NCX activity, so reperfusions with high Na^+ solution, low Ca^{2+} solution, low pH solution, and solution with inhibitor of NCX, have been

* Corresponding author. Department of Physiology, Fourth Military Medical University, 169# Changle West Road, Xi'an 710032, China. Tel.: +86 29 84774519; fax: +86 29 84776423.
E-mail address: jmpei8@fmmu.edu.cn (J.-M. Pei).

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reported to improve contractile function of myocytes (du Toit and Opie, 1992; Hendrikx et al., 1994; Kitakaze et al., 1988; Kusuoka et al., 1993; Tani and Neely, 1990). Recently, inhibition of the reverse mode of NCX has been shown to improve contractile function (Takahashi et al., 2004). These above mentioned findings suggest that during ischemia and early reperfusion Ca^{2+} overload induced by NCX could contribute to stunning. Although research in pathogenesis of myocardial stunning makes progress, the factors, which result in prolonged reduction of contractile function following brief ischemia, remain to be elucidated. One important point is whether both or one of systolic and diastolic Ca^{2+} overload is involved in the causing of myocardial stunning. Another point is that: some reports show that intracellular Ca^{2+} does not rise during ischemia less than 15 min (Ladilov et al., 1995; Tani and Neely, 1989). If so, could post-ischemic dysfunction occur or not, and if it occurs, what is the mechanism? In addition, inhibition of L-type Ca^{2+} channel has also been reported to reduce myocardial stunning during the period of ischemia and early reperfusion in non-cell model (du Toit and Opie, 1992; Smart et al., 1997). And whether NCX and/or L-type Ca^{2+} channel could result in myocardial contractile dysfunction in brief ischemia/reperfusion cell model is the third question we are trying to answer.

Most of the previous studies of myocardial stunning have been performed in vivo or in isolated hearts. However, in order to elucidate this cellular mechanism, it is necessary to have an isolated cell model of myocardial stunning. Accordingly, the first objective was to confirm whether myocardial stunning could be observed in isolated cardiomyocyte. The second goal

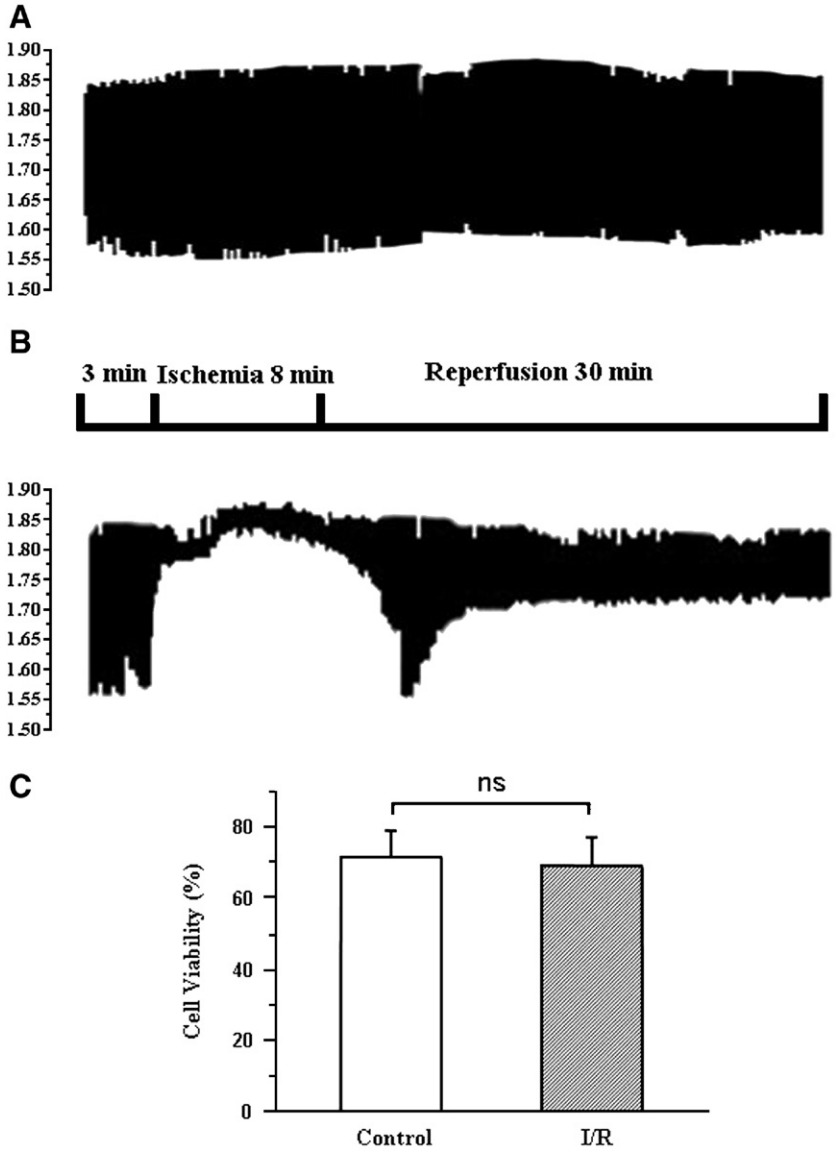

Fig. 1. Myocardial stunning model in isolated myocytes and the effect of low Ca^{2+}. A and B, Representative recordings of changes in cell contraction in control group and I/R group. C, Cell viability in control group and I/R group. $n=6$. ns, no significant difference. D, Representative recordings of cell contraction in I/R group (top) and I/R + Low Ca^{2+} group (bottom) at different time. E, Statistical results of effect of low Ca^{2+} on cell contraction at the end of reperfusion. Contraction was normalized as a percent of pre-ischemic values. $^{**}P<0.01$ *vs* I/R group. $n=9$ from 6 rats.

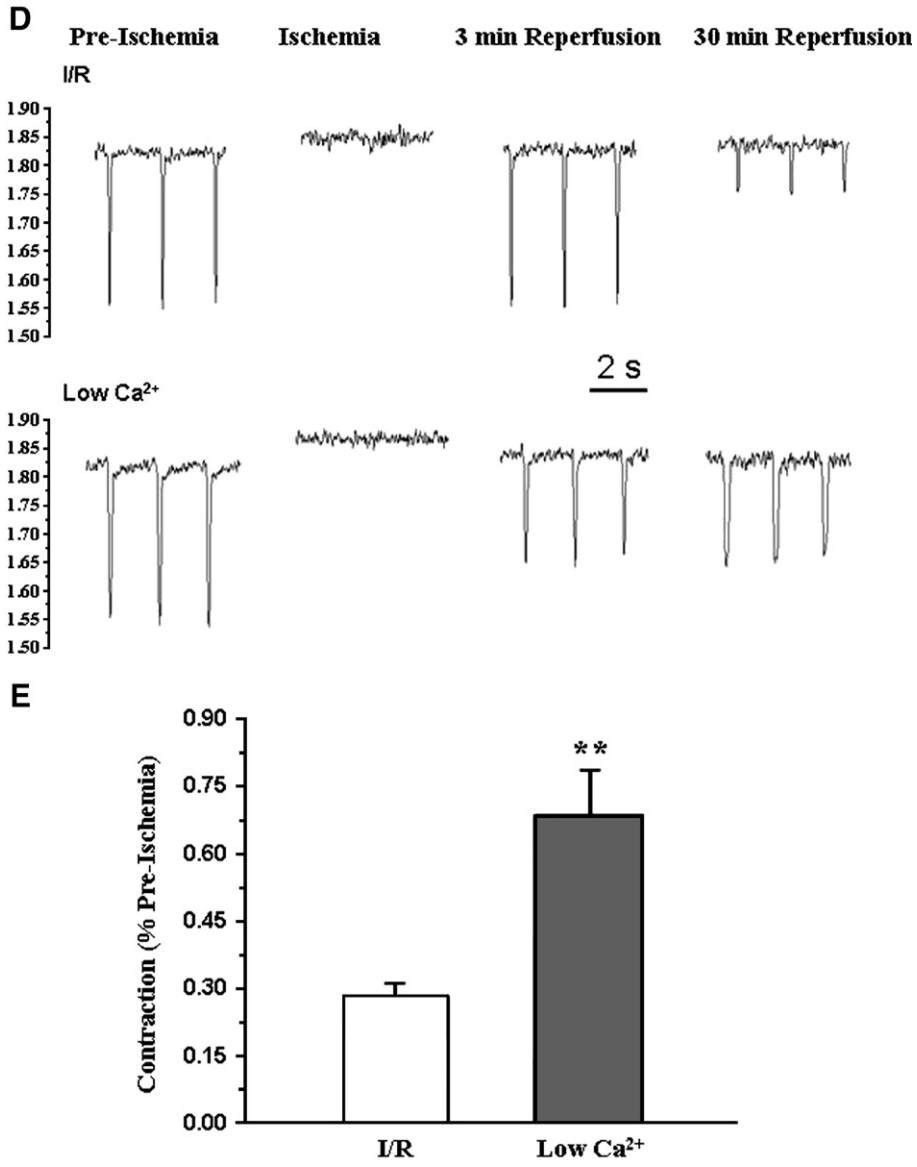

Fig. 1 (*continued*).

was to determine whether diastolic Ca^{2+} overload during early reperfusion could cause myocardial stunning even if brief ischemia does not induce Ca^{2+} overload. The final goal was to determine which factors could lead to diastolic Ca^{2+} overload.

2. Material and methods

2.1. Isolation of ventricular myocytes

The experiments were performed in adherence to the National Institutes of Health Guidelines for the use of Laboratory Animals and were approved by the Fourth Military Medical University Committee on Animal Care. Ventricular myocytes were isolated from male Sprague–Dawley rats (220–250 g), by a collagenase perfusion method described previously (Zhou et al., 2002). Briefly, after the hearts were digested by collagenase for 25–30 min, the left ventricular tissue was cut into small pieces in high K^+ solution containing (in mM) 10 KCl, 10 KH_2PO_4, 120 K-glutamate, 10 taurine, 1.0 $MgSO_4$, 10 HEPES, 20 glucose, and 0.5 EGTA (adjusted to pH 7.2 with KOH). After a gentle stirring for 5 min, the residue was filtered through a 250 μm mesh screen. Myocytes were stored in high K^+ solution for 40 min, and then re-suspended in normal Tyrode solution with 1% BSA. Only cells exhibiting a rod-shaped morphology and no signs of sarcolemmal blebbing were used for the experiments. Isolated cells were all used for experiments within 6 h after isolation.

2.2. Measurements of cell shortening and intracellular Ca^{2+}

Myocytes were placed in a 0.3 ml microperfusion chamber mounted on an inverted microscope (IX 50, Olympus, Tokyo, Japan), and perfused at 1.5 ml/min. Myocyte contraction was induced at 0.5 Hz by a pair of platinum field electrodes. Cell shortening was assessed with a video-based motion edge-

detection analyzer. Intracellular Ca^{2+} was measured by the fluorescent calcium indicator Fura-2 in a dual-fluorescence, calcium ion-sensing system (IonOptix, Milton, MA). Cells were loaded 5 μM Fura-2/AM for 30 min as described previously (Dong et al., 1993; Zhou et al., 2002). Fluorescent signals obtained at 340 nm (340) and 380 nm (380) excitation wavelengths were recorded and stored in a computer for data processing and analysis. The 340/380 ratio was used to represent cytosolic Ca^{2+} in the ventricular myocytes.

2.3. Simulated ischemia/reperfusion in isolated myocytes

Ischemia was accomplished with glucose-free Tyrode solution (pH 6.4) containing 10 mM 2-deoxy-D-glucose (DOG), an inhibitor of glycolysis, and 2 mM sodium dithionite, an oxygen scavenger, known to cause acidosis, metabolic inhibition and anoxia (Chen et al., 2003; Ho et al., 2002). After 8 min of ischemia, the myocytes were reoxygenated with normal Tyrode solution for 30 min. During the first 10 min of reperfusion myocytes were subjected to different treatments such as adding KB-R7943 etc.

2.4. Measure of reverse-mode NCX activity and trypan blue exclusion

After the first 3 min of reperfusion, myocytes were exposed to Na^+-free solution for 1 min, then reperfused with normal Tyrode solution to determine the reverse-mode NCX activity (Mochizuki and MacLeod, 1997). At the end of the 30 min reperfusion myocyte viability was estimated by Trypan blue exclusion (Zhou et al., 2001). The cells were incubated with 0.4% trypan blue for 2 min, and approximately 200 cells in each group were examined in a hemocytometer chamber under a light microscope. Cells able to exclude the stain were considered viable and the percentage of unstained cells over total cells provided an index of viability.

2.5. Chemicals

The normal Tyrode solution contained (in mM): 140 NaCl, 4.2 KCl, 1.2 $MgCl_2$, 1.25 $CaCl_2$, 1.2 KH_2PO_4, 10 glucose, and 10 HEPES; pH was adjusted to 7.4 with NaOH. The low Ca^{2+} Tyrode solution was similar to normal Tyrode solution, except that concentration of $CaCl_2$ was 0.5 mM. The Na^+-free Tyrode solution was similar to normal Tyrode solution, except that NaCl was substituted for 125 mM N-methyl-D-glucamine (NMDG).

DOG, type I collagenase, HEPES, and Fura-2/AM were purchased from Sigma. 2-[2-[4-(4-Nitrobenzyloxy)phenyl] ethyl]isothiourea mesylate (KB-R7943) was from Tocris.

All chemicals were dissolved in distilled water except Fura-2/AM, nifedipine and KB-R7943, which were dissolved in DMSO at a final concentration of <0.1%.

2.6. Statistical analysis

Data are presented as mean ± standard error of mean (mean ± S.E.M.). One-way ANOVA and the post hoc Tukey's test were used for multiple comparisons at a minimal significance level of $P<0.05$. Student's *t* test was used when it is applicable to simple two-sample tests at the same minimal significance level.

3. Results

3.1. Establishment of myocyte model of myocardial stunning

In order to investigate the mechanism underlying myocardial stunning, we adopted the method firstly described by Louch et al. (2002) and modified an isolated cellular model of myocardial stunning, which permits to easily measure cell shortening and cytosolic Ca^{2+} concentrations.

Fig. 1A showed representative recordings of cell contractions during the whole period of experiments, and the whole duration lasted about 40 min. Time course of control recordings without ischemia/reperfusion (I/R) was shown in Fig. 1A, and cell shortening did not change markedly under control conditions during the whole experiment. Cell shortening was quickly decreased and even abolished when the cell was subjected to the 8 min of ischemia (Fig. 1B). In early reperfusion, contraction recovered gradually to pre-ischemic levels after 3 min of reperfusion. However, after 4–5 min of reperfusion, myocyte contraction was significantly depressed, and this significant reduction in cell shortening continued for the rest time of experiment (Fig. 1B, D).

In addition, according to previous definition, myocardial stunning occurs in the absence of irreversible damage during reperfusion after restoration of normal coronary flow (Bolli, 1990), we also determined if myocytes exhibited a significant change in cell viability due to ischemia/reperfusion after 30 min reperfusion compared with control. It was found that reperfusion with normal solution following brief ischemia did not significantly increase cell death at the end of a 30 min reperfusion compared with control (Fig. 1C). Moreover, it was observed that cells did not have contracture or hypercontracture during the period of ischemia and reperfusion. Thus, under the present procedure, this model was suitable to determine and evaluate myocardial stunning.

3.2. Effect of reperfusion with low Ca^{2+} on myocardial stunning

In the following series of experiments, 0.5 mM of Ca^{2+} buffer was used at the first 10 min of reperfusion to determine the effect of low Ca^{2+} on myocardial stunning.

Fig. 1D showed representative recording of cell shortening at selected time points. Cell contractions of both groups were completely inhibited during ischemia. In I/R group, at the first 3 min of reperfusion cell shortenings nearly recovered to pre-ischemic levels; however, after 3 min of reperfusion cell shortenings rapidly decreased and kept at relatively lower stable levels (Fig. 1B). And cell shortenings were markedly depressed at the end of 30 min's reperfusion compared with pre-ischemic levels (Fig. 1D top). Because of cells exposed to external low Ca^{2+} buffer, contractions did not recover to pre-ischemic levels at the first 3 min of reperfusion in I/R + Low Ca^{2+} group compared with I/R group (70.2 ± 0.67% of pre-ischemia). However, after reperfusion buffer was changed into normal concentration of

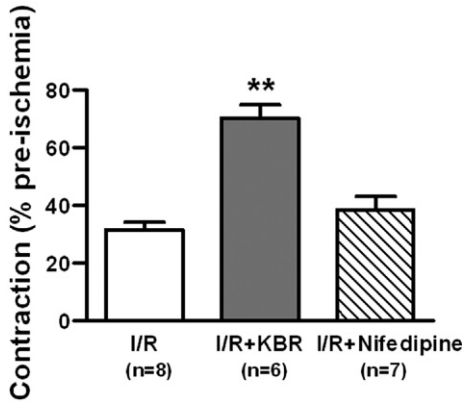

Fig. 2. Effect of KB-R7943 and nifedipine on cell contraction at the end of reperfusion. Contraction was normalized as a percent of pre-ischemic values. **$P<0.01$ *vs* I/R group. KBR, KB-R7943.

Ca^{2+} buffer following 10 min of low Ca^{2+} buffer reperfusion, myocytes exhibited a good recovery of cell shortenings at the end of a 30 min reperfusion compared with I/R group (Fig. 1D bottom). We also got the statistical results in Fig. 1E and found that cell contraction got more significant restoration in I/R+Low Ca^{2+} group than that in I/R group ($P<0.01$). Therefore, decreasing Ca^{2+} entry into myocardial cells at the first 10 min of reperfusion was a very efficient way to prevent myocardial stunning caused by brief ischemia and reperfusion.

3.3. Effects of the reverse mode of NCX and L-type Ca^{2+} channel on myocardial stunning

Extracellular Ca^{2+} enters the cells mainly through two pathways, they are the reverse mode of NCX pathway and L-type Ca^{2+} channel pathway, respectively. So we tried to determine if the two pathways were involved in myocardial stunning.

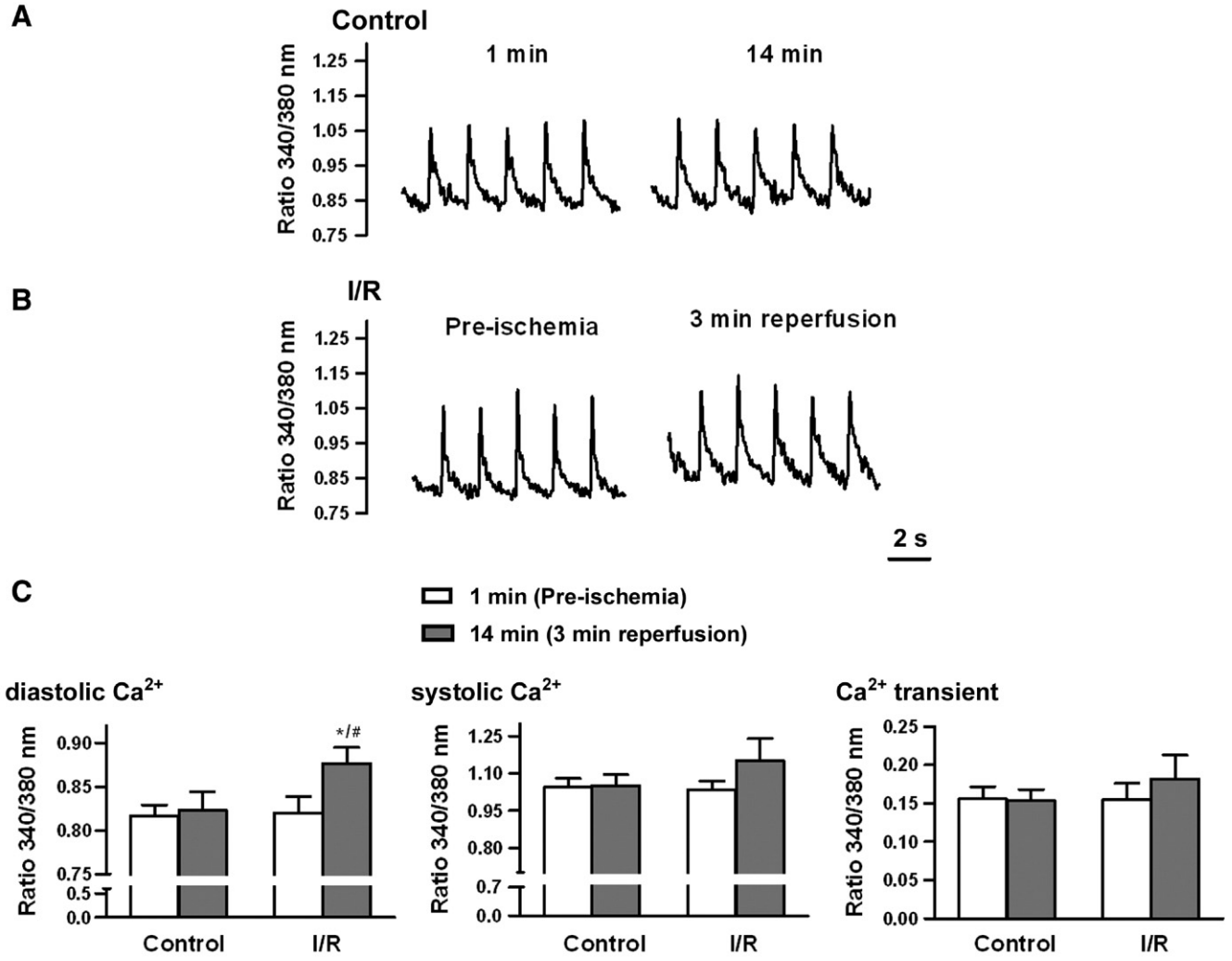

Fig. 3. Changes in intracellular Ca^{2+} in groups with different treatments. A and B, Representative recordings of intracellular Ca^{2+} at different time were exhibited in control group and I/R group, respectively. C, Statistical results of Ca^{2+} transient, systolic Ca^{2+} and diastolic Ca^{2+}. *$P<0.05$ *vs* control, #$P<0.05$ *vs* pre-ischemia. $n=9$. D, Representative results of intracellular Ca^{2+} changes with different treatments at different time. E F G, Statistical results of systolic Ca^{2+}, diastolic Ca^{2+} and Ca^{2+} transient, respectively. Intracellular Ca^{2+} was normalized as a percent of pre-ischemic values. *$P<0.05$,**$P<0.01$ *vs* I/R group; #$P<0.05$ *vs* pre-ischemia. $n=9$.

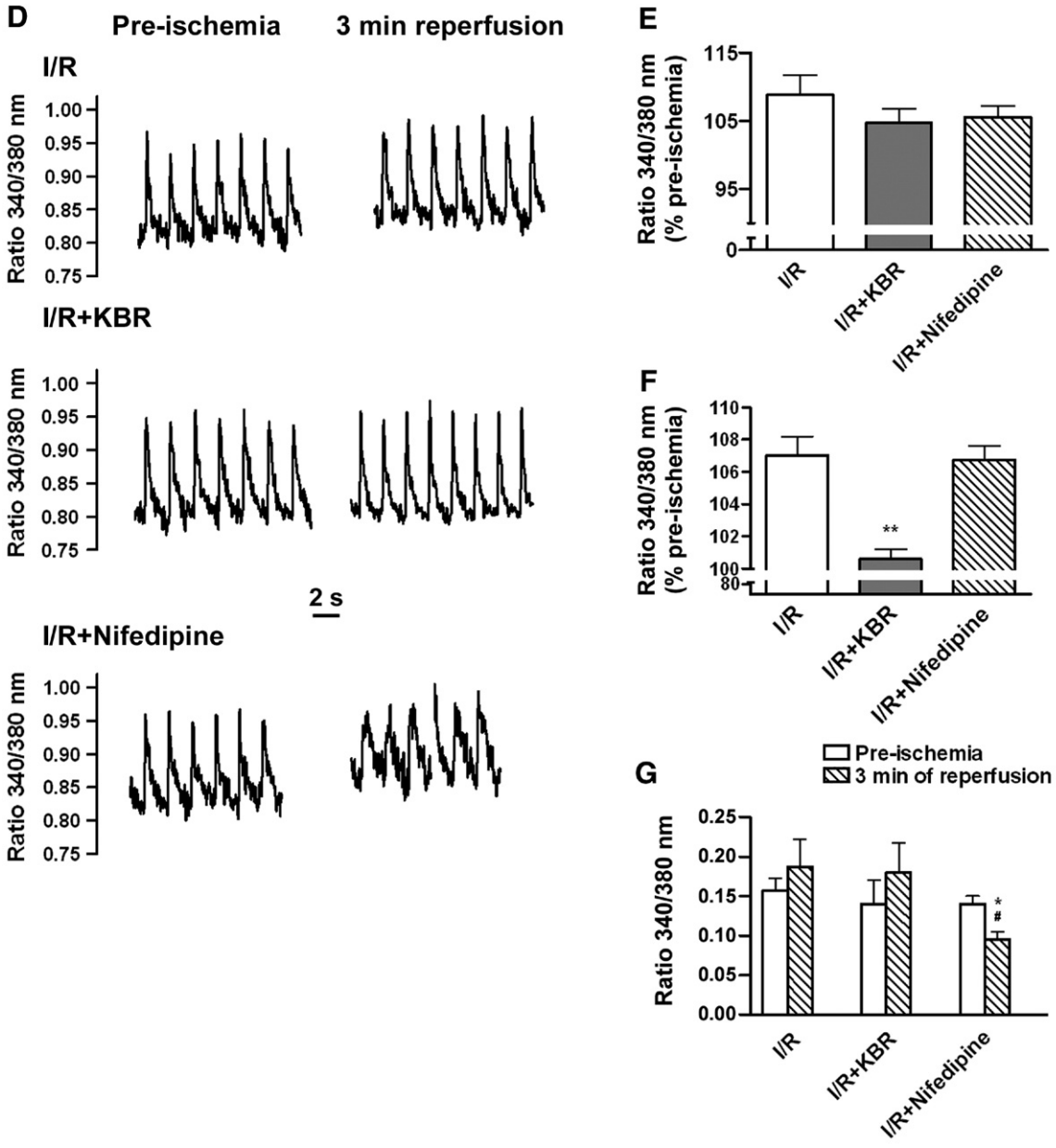

Fig. 3 (*continued*).

KB-R7943, a selective inhibitor of the reverse mode of NCX, at the concentration of 5 μM (Satoh et al., 2000), was administered at first 10 min of reperfusion significantly attenuated the myocyte contractile depression at the end of a 30 min reperfusion (Fig. 2). However, nifedipine, a selective inhibitor of L-type Ca^{2+} channel, at the concentration of 1 μM (Marengo et al., 1998; Maruyama et al., 2006), when used at first 10 min of reperfusion, failed to improve the myocytes contractile functions at the end of reperfusion (Fig. 2).

3.4. Role of diastolic Ca^{2+} overload during the initial period of reperfusion in myocardial stunning

To study the mechanism myocardial stunning, we first determined intracellular Ca^{2+} without any treatment during the period of ischemia and reperfusion. If cardiomyocytes were not exposed to ischemia, there were no changes in the electrically induced Ca^{2+} transient, and systolic Ca^{2+} and diastolic Ca^{2+} in the period of protocols (Fig. 3A, C). However, when cardiomyocytes were exposed to 8 min's simulated ischemia, the amplitude of Ca^{2+} transient gradually decreased to zero (the ratio was 0.834± 0.021). In the initial period of reperfusion Ca^{2+} transient recovered rapidly, and systolic Ca^{2+} and Ca^{2+} transient surpassed those at pre-ischemia but with no statistical significance compared with pre-ischemia. However, intracellular diastolic Ca^{2+} significantly increased compared with pre-ischemia and control, and it was up to the maximum at the time point of about 3 min during reperfusion (Fig 3B, C).

We then determined intracellular Ca^{2+} during the period of ischemia and reperfusion when myocytes were exposed to KB-

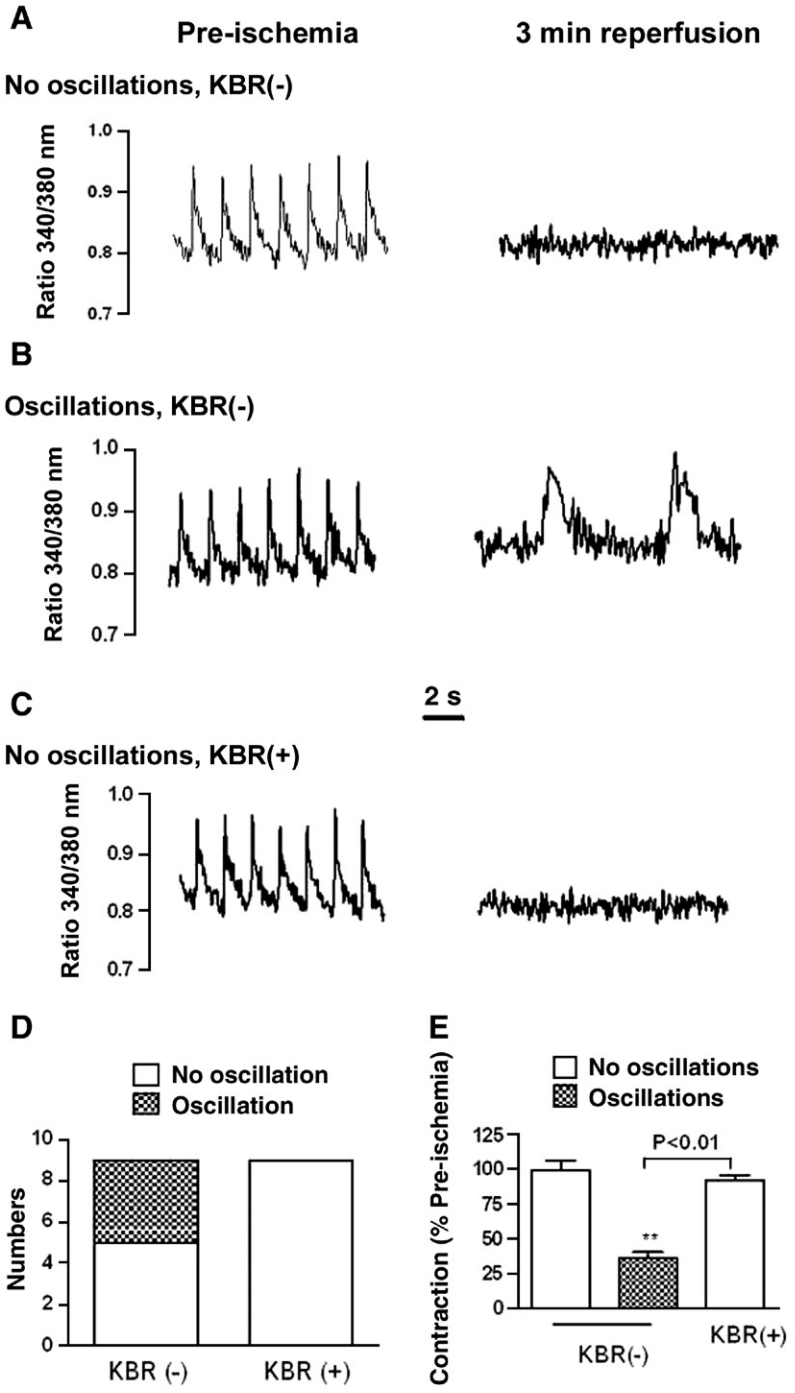

Fig. 4. Intracellular Ca^{2+} changes with no stimulus at the first 10 min reperfusion and subsequent effect on contraction in the absence or in the presence of KB-R7943. A B, With or without oscillations in the absence of KB-R7943. C, No oscillations occurred in the presence of KB-R7943. D, Statistical results of intracellular Ca^{2+} changes at 3 min during reperfusion. E, Contractions with different treatments after 30 min of reperfusion. Contraction was normalized as a percent of pre-ischemic values. $^{**}P<0.01$ *vs* no oscillations group. KBR. KB-R7943

R7943 or nifedipine at the first 10 min reperfusion. The first exposure of myocytes to KB-R7943 in the early reperfusion entirely inhibited diastolic Ca^{2+} increase compared with I/R group ($P<0.01$), but did not change systolic Ca^{2+} and amplitude of Ca^{2+} transient (Fig. 3D–G), with an improved contractile function in the later reperfusion (Fig. 2). However, when myocytes were subjected to nifedipine in the first 10 min reperfusion, changes in systolic Ca^{2+} and diastolic Ca^{2+} were similar to those in I/R group, except that Ca^{2+} transient in nifedipine administered group was attenuated more significantly than that in I/R group (Fig. 3 D–G). Together with the above mentioned result that nifedipine had no effect on myocardial stunning in the later reperfusion (Fig. 2), these results indicated that diastolic Ca^{2+} overload at first 10 min

reperfusion following brief ischemia played an important role in the occurrence of myocardial stunning in the later long-time reperfusion.

3.5. NCX, but not L-type Ca^{2+} channel involved in protection against myocardial stunning caused by brief ischemia and reperfusion

In the next series of experiments, we stopped field stimulus in the first 10 min reperfusion to determine intracellular Ca^{2+} and cell contraction with no involvement of L-type Ca^{2+} channel. Interestingly, when electrical stimulus was not used in the first 10 min reperfusion, there were two different changes in intracellular Ca^{2+}, namely, Ca^{2+} oscillations (4 cells/9 cells) and no Ca^{2+} oscillations (5 cells/9 cells) in the early 10 min of reperfusion (Fig. 4A, B, D). If no Ca^{2+} oscillation occurred in this series of experiments, cell contraction recovered to pre-ischemic level at the end of a 30 min reperfusion, but if there were Ca^{2+} oscillations cell contraction could not be significantly improved (Fig. 4E). When 5 μM of KB-R7943 was added into reperfusion buffer, there was only the phenomenon of no Ca^{2+} oscillations observed during first 10 min reperfusion in 9 cells from 6 rats (Fig. 4C, D). Moreover, 5 μM of KB-R7943 protected cell from contractile dysfunction in the later reperfusion (Fig. 4E). Therefore, these results indicated that NCX, not L-type Ca^{2+} channel, was involved in protection against myocardial stunning caused by brief ischemia and reperfusion.

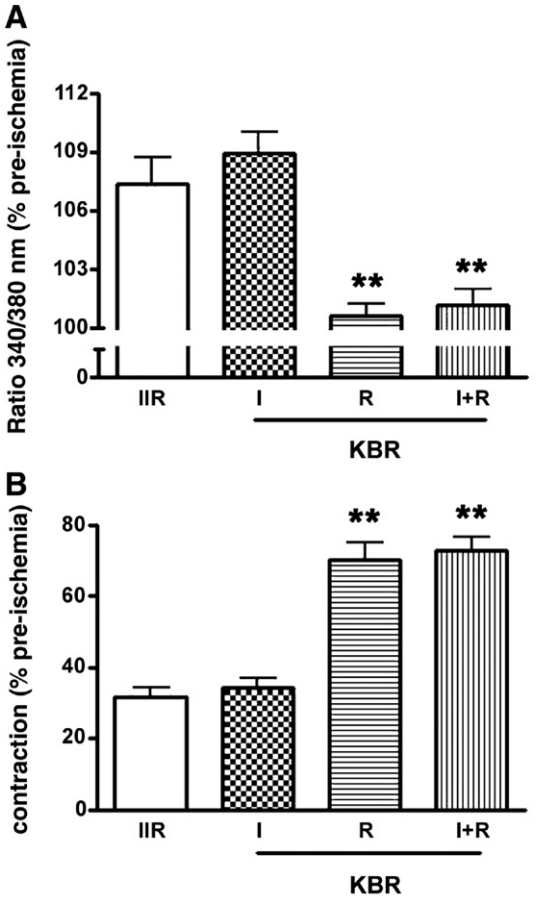

Fig. 5. Effect of KB-R7943 on diastolic Ca^{2+} (A) at 3 min during reperfusion and contraction (B) at the end of reperfusion during different time points of administration. Intracellular Ca^{2+} and contraction were normalized as a percent of pre-ischemic values. I/R, no treatment during ischemia and reperfusion; KBR, with treatment of KB-R7943 only during ischemia (I), only during the first 10 min of reperfusion (R), and from initial time of ischemia to 10 min of reperfusion (I+R). $^{**}P<0.01$ *vs* I/R; $n=6$.

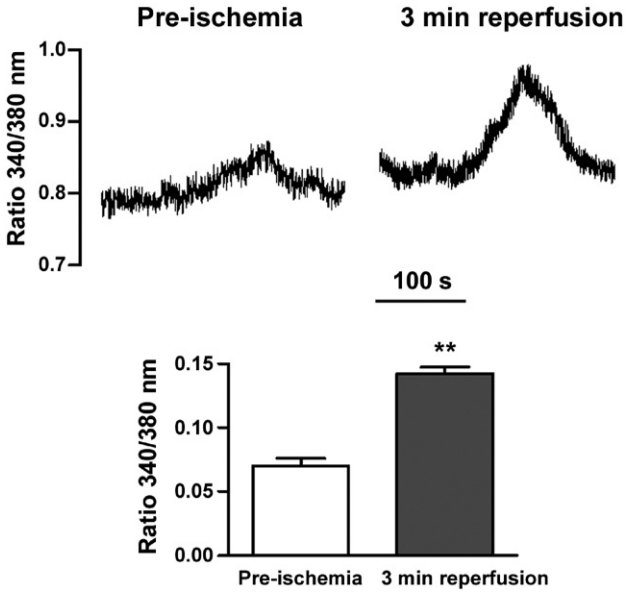

Fig. 6. The activity of the reverse mode of NCX at the first 3 min during reperfusion. The ratio of 340/380 nm was 0.07±0.006 in pre-ischemia group and 0.14±0.005 in 3 min reperfusion group, respectively. $^{**}P<0.01$ *vs* Pre-Ischemia; $n=8$.

3.6. Diastolic Ca^{2+} and contraction at different time of using KB-R7943

We observed the effect of KB-R7943 on myocardial stunning during different periods of administration. As shown in Fig. 5, when KB-R7943 was administrated only during brief ischemia, diastolic Ca^{2+} overload in myocytes was not inhibited in the early phase of reperfusion (Fig. 5A) and contractile dysfunction did not improve at the end of a 30 min reperfusion (Fig. 5B). If KB-R7943 was administrated from initial period of ischemia to 10 min of reperfusion or only during the early 10 min of reperfusion, diastolic Ca^{2+} overload in myocyte did not occur, which conferred protection against myocardial stunning in the late period of reperfusion (Fig. 5).

3.7. Measurements of the reverse mode of NCX activity

In the final experiments, we determined the reverse mode of NCX activity by Na^{+}-free methods. The activity of the reverse mode of NCX was significantly higher at 3 min post-ischemic reperfusion than that in the pre-ischemia period (Fig. 6).

4. Discussion

In this paper we used isolated cell model to study the pathogenesis of myocardial stunning caused by brief ischemia.

We first established the cell model to observe the phenotype of myocardial stunning, and at the same time to determine intracellular Ca^{2+} and cell morphologic change. Secondly, we found that diastolic Ca^{2+} overload in the initial period of reperfusion, which could occur even though intracellular Ca^{2+} did not increase during brief ischemia, resulted in subsequent myocardial stunning at the end of reperfusion. Thirdly, we found that diastolic Ca^{2+} overload was caused by abnormal enhancement of the activity of the reverse mode of NCX in the initial period of reperfusion following brief ischemia.

In the present study, to investigate the pathogenesis of myocardial stunning, we developed a modified cell model of cardiac stunning in rats. In the previous studies, in order to investigate the mechanism of ischemia/reperfusion injury, the myocytes were subjected to long-time serious simulated ischemia. Because long-time ischemia causes serious damage and death of myocytes, the ischemic time was reduced to 8 min in the present protocol, which does not influence the cell length in the recording process. Moreover, when cells were subjected to brief ischemia for 8 min, cell viability was not significantly different from that in control group (Fig. 1). Thus, the myocytes model can be adopted to investigate myocardial stunning. Louch et al. have reported an isolated myocytes model (Louch et al., 2002). In their protocol, ischemic time is 30 min, and during the period of ischemia the phenomenon that the cell length becomes short, i.e. rigor-contracture, could be observed. However, it was observed that if rigor-contracture occurred during ischemia, cardiomyocytes became more fragile and susceptible to irreversible damage during reperfusion. Therefore, to avoid unnecessary interference of some factors, we adopted the present protocol to prevent the occurrence of rigor-contracture during ischemia.

In the present study, by using this stunning model we showed that diastolic Ca^{2+} overload at the first 10 min of reperfusion plays a crucial role in triggering myocardial stunning in the later period of reperfusion. And another important finding is that attenuation of Ca^{2+} transient during the initial period of reperfusion is not involved in improving contractile dysfunction of myocytes. Previous findings have confirmed that decrease in Ca^{2+} overload protects cardiomyocytes exposure to prolonged serious ischemia against ischemia/reperfusion injury (Inserte et al., 2002; Stromer et al., 2000; Tani and Neely, 1989). In the present experiment, we demonstrated that myocytes exhibit significant diastolic Ca^{2+} overload during the initial period of reperfusion although they are exposed to brief ischemia. If diastolic Ca^{2+} overload is inhibited during reperfusion as quickly as possible, myocytes may make a good improvement on contractile dysfunction at the end of reperfusion. We also showed that Ca^{2+} overload does not happen during a brief period of ischemia in the present protocol although it happens during the first 10 min of reperfusion. The result is in agreement with previous reports, which have shown that only a long-time period of ischemia leads to Ca^{2+} overload (Ladilov et al., 1995; Schafer et al., 2000; Siegmund et al., 1992; Tani and Neely, 1989, 1990). These suggest that Ca^{2+} overload during reperfusion would indirectly result from the changes of some factors caused by a brief ischemia, such as intracellular H^+ and Na^+ accumulations, etc. Accumulations of intracellular H^+ and Na^+ have been reported to occur even in the early period of ischemia in some papers, and intracellular Na^+ remains significantly elevated for 8–10 min after reflow (Ladilov et al., 1995; Schafer et al., 2000; Tani and Neely, 1989; van Emous et al., 1998). The elevation in intracellular Na^+ may activate the reverse mode of NCX, which in turn leads to the Ca^{2+} overload during the early reperfusion. Therefore, myocardial contractile dysfunction was not improved when the inhibitor of NCX was administered only during brief ischemia in the present experiment.

In this study, we found that inhibition of the reverse mode of NCX during the early period time of reperfusion prevents cells from inducing diastolic Ca^{2+} overload, and thereafter improves the contractile dysfunction of myocytes. However, nifedipine, which reduces the amplitude of Ca^{2+} transient but does not reduce diastolic Ca^{2+} overload during the first several minutes of reperfusion, does not protect myocytes from subjected to myocardial stunning. Moreover, we designed a protocol for further observation of the effect of NCX on myocardial stunning without involvement of L-type Ca^{2+} channel, in which no field stimulus is performed during the first 10 min of reperfusion. The results showed that there are two phenomena, i.e. with or without Ca^{2+} oscillation, and if Ca^{2+} oscillation does not occur, myocytes hardly suffer from stunning; on the contrary, if Ca^{2+} oscillation occurs myocytes confront with contractile dysfunction in the late reperfusion. Previous report has confirmed that Ca^{2+} oscillations are tightly correlated with the activation of the reverse mode of NCX (Schafer et al., 2001). Therefore, the inhibitor of the reverse mode of NCX is used during the first 10 min of reperfusion. The result shows that Ca^{2+} oscillations do not occur with the treatment of KB-R7943, and myocardial stunning is not found at the end of reperfusion accordingly. In the end, the activity of the reverse mode of NCX at the time of 3 min during reperfusion is found to be abnormally enhanced, which further confirms that activation of the reverse mode of NCX during the initial period of reperfusion leads to diastolic Ca^{2+} overload, and thereafter confers myocardial stunning. Because the reverse mode of NCX is mediated by transsarcolemmal Na^+ gradient, and previous researches have directly demonstrated that cytosolic Na^+ overload occurs during the initial period of ischemia and remains significantly increasing for about 10 min during reperfusion (Ladilov et al., 1995; Schafer et al., 2000; van Emous et al., 1998), it is speculated that the accumulation of intracellular Na^+ during ischemia and reperfusion contributes to the abnormal activation of the reverse mode of NCX. In addition, Ca^{2+} oscillation did not occur in all myocytes with no field stimulus, which suggested that electrical stimulus could play an important role in activating the reverse mode of NCX during reperfusion following ischemia. There could be two reasons: 1) the activity of the reverse mode of NCX increases when membrane potential becomes more positive than reversal potential of NCX during cardiac action potential; 2) Na^+ entry induced by Na^+ channel promotes reverse-mode activity of NCX during action potential (Leblanc and Hume, 1990; Weber et al., 2002).

In addition, it is well known that depression of myofilament Ca sensitivity is an important reason for the stunning, although

we did not observe this parameter in our present study, We believe that calcium overload induced by NCX in the early reperfusion is probably co-existed with the depression of myofilament Ca sensitivity.

In clinic, thrombolytic therapy and percutaneous transluminal coronary angioplasty, etc. are extensively adopted to treat patients with coronary artery diseases. As a result, myocardial stunning has become to be quite common phenomenon in the management of acute ischemic syndromes, and therefore prevention of contractile dysfunction is very important for recovery of patients after therapy. Our results show that diastolic Ca^{2+} overload due to the reverse mode of NCX triggers myocardial stunning. And our present research also provides some insight into the prevention of myocardial stunning in the management of acute ischemic syndromes. Reperfusion with low Ca^{2+} buffer is an appropriate treatment during the initial period of reperfusion. Treatment with an inhibitor of the reverse mode of NCX during the early time of reperfusion is probably another option. Of course, it is optimal method that myocardial contraction is temporarily inhibited in the procedure of treatment with an inhibitor of the reverse mode of NCX.

In summary, we have successfully established an isolated cell model of myocardial stunning. Our results provide the clear, direct evidence in this model to demonstrate that diastolic Ca^{2+} overload caused by the reverse mode of NCX rather than L-type Ca^{2+} channel during the first several minutes of reperfusion after brief ischemia is one of the important mechanisms for triggering myocardial stunning even though brief ischemia could not cause intracellular Ca^{2+} overload. We also show that the initial period of reperfusion is an important target for protecting myocardium against contractile dysfunction.

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