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L-*cis* Diltiazem attenuates intracellular Ca^{2+} overload by metabolic inhibition in guinea pig myocytes

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Abstract

We have previously demonstrated that treatment with L-*cis* diltiazem reduced cardiac infarct size in vivo. To examine the effect of L-*cis* diltiazem on Ca^{2+} overload induced by ischemia/reperfusion, we used a model for Ca^{2+} overload produced by metabolic inhibition in isolated guinea pig myocytes. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was quantified by fura-2 fluorescence microscopy and Ca^{2+} overload was induced by inclusion of 1 μM of carbonyl cyanide *m*-chrolophenylhydrazone (CCCP) for 40 min treatment followed by washout for 30 min. This treatment caused a large $[Ca^{2+}]_i$ elevation as well as a sustained contracture of the cardiomyocytes. The increase was suppressed by 10 μM of 2-[2-[4-(4-nitrobenzyloxy) phenyl] ethyl] isothiourea methanesulphonate (KB-R7943), a specific inhibitor of the Na^+/Ca^{2+} exchanger, but not by nitrendipine (10 μM). L-*cis* Diltiazem (10 μM) attenuated the $[Ca^{2+}]_i$ increase, suggesting that L-*cis* diltiazem elicits a cardioprotective effect via attenuation of the $[Ca^{2+}]_i$ increase induced by metabolic inhibition and energy repletion.

Keywords: Metabolic inhibition; Ca^{2+}; L-*cis* Diltiazem; KB-R7943; (Guinea pig); Cardiac myocyte

1. Introduction

L-*cis* Diltiazem, the enantiomer of diltiazem (D-*cis* diltiazem), has 100 times less Ca^{2+} channel blocking activity than diltiazem (Ikeda et al., 1991). L-*cis* Diltiazem is not a simple inactive stereoisomer but has been established as a unique pharmacological tool (Caretta et al., 1979). For example, L-*cis* diltiazem, but not diltiazem, inhibits the cGMP-gated cation channel in rod cells (Stern et al., 1986; Quandt et al., 1991). Interestingly, there have been several reports indicating that L-*cis* diltiazem protects against ischemic cardiac injury. For example, L-*cis* diltiazem produces a high recovery of cardiac function after ischemia/reperfusion and inhibits the increase of non-esterified fatty acids during ischemia in isolated, perfused working rat heart (Nasa et al., 1990; Xiao et al., 1997).

Recently, our laboratory has reported that treatment with L-*cis* diltiazem reduced infarct size without affecting any hemodynamic parameter in rabbit heart in vivo (Nishida et al., 1999). Since L-*cis* diltiazem has a much less potent Ca^{2+} channel blocking action than diltiazem, it has been suggested that L-*cis* diltiazem protects against ischemia/reperfusion injury with a mechanism other than Ca^{2+} channel blockade.

We have also demonstrated that treatment with L-*cis* diltiazem before reperfusion also reduced infarct size in ischemic rabbit heart (Nishida et al., 1999). Ca^{2+} overload at reperfusion after ischemia (Allen and Orchard, 1983) and reoxygenation after hypoxia (Poole-Wilson et al., 1984) have been considered to be involved in the final stage of ischemia/reperfusion injury of cardiac myocytes (Nayler, 1981; Farber, 1982; Steenbergen et al., 1990). Thus, it is postulated that L-*cis* diltiazem elicits a cardioprotective action via the attenuation of Ca^{2+} overload after reperfusion.

In terms of energy metabolism, ischemic insults can be simulated, in part, by controlled metabolic inhibition. Li et al. (1989) has reported that the myocytes were reversibly

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ATP-depleted by metabolic inhibitors. Thus, myocardial Ca^{2+} overload induced by ischemia and reperfusion is considered to be simply mimicked, in part, by treatment with carbonyl cyanide *m*-chrolophenylhydrazone (CCCP), a mitochondrial uncoupler, followed by washout of CCCP.

In order to assess the effect of L-*cis* diltiazem on reperfusion-induced Ca^{2+} overload, we used a model for Ca^{2+} overload by metabolic inhibition and energy repletion in isolated guinea pig cardiomyocytes. We also assessed the effect of other drugs, Ca^{2+} channel blockers and Na^+/Ca^{2+} exchanger blockers, to clarify the mechanism for Ca^{2+} overload in this model.

2. Materials and methods

2.1. Preparation of single cells

Ventricular myocytes were isolated enzymatically from the hearts of male Hartley guinea pigs (weight 250–500 g) according to the method described (Kurokawa et al., 1997). The animals were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the ascending aorta was cannulated in situ under artificial respiration. The heart was perfused with Ca^{2+}-free Tyrode solution for 10 min, followed by the same solution containing collagenase for 7–14 min at a perfusion rate of 8–10 ml/min via a Langendorff apparatus at 37°C. Subsequently, the enzyme solution was washed out with a high K^+, Ca^{2+}-free solution (Kraftbrühe (KB) solution, Isenberg and Klöckner, 1982). The ventricular myocytes were dissociated by gentle stirring of the tissue fragments at 37°C. The dissociated cells were stored in KB solution at 4°C, and were used up to 10 h after isolation. Nominally Ca^{2+}-free solution had the following composition (mM): NaCl 135, KCl 5.4, $MgCl_2$ 1, glucose 5.8, HEPES 5 (pH 7.4, adjusted with tris). Normal Tyrode solution was made by adding 1.8 mM $CaCl_2$ to nominally Ca^{2+}-free solution. The collagenase solutions were prepared by adding collagenase (110 units/ml Collagenase S-1, Nitta Gelatin, Osaka, Japan; 200–220 units/ml collagenase, Yakult, Tokyo. Japan) and 10.8 nM $CaCl_2$ to Ca^{2+}-free Tyrode solution, or by combining collagenase (0.145 U/ml collagenase A, Boehringer Mannheim, Japan) and protease (0.156 units/ml protease type IX, Sigma, St. Louis, MO, USA) to Ca^{2+}-free Tyrode solution. The KB solution contained (mM): potassium glutamate 70, KCl 20, oxalic acid 10, KH_2PO_4 10, taurine 10, glucose 5.8, HEPES 5, EGTA 0.5 (pH 7.4, adjusted with KOH).

2.2. Measurement of fluorescence ratio of fura-2 in cardiac myocytes

For measurement of a change in $[Ca^{2+}]_i$ of cardiac myocytes, a Ca^{2+}-sensitive fluorescent dye, fura-2/acetoxymethylester (fura-2/AM), was used. Fura-2/AM (1 mM, dissolved in dimethylsulfoxide, DMSO) was prepared in KB solution and applied to isolated myocytes at a final concentration of 10 μM. After the cell suspension had been incubated for 10 min at 37°C, the cells settled out and became loosely attached to the bottom of the chamber. Then the fura-2-loaded myocytes were washed and superfused (4–5 ml/min, 37°C) with normal Tyrode solution containing 1.8 mM Ca^{2+} for 30 min. The myocytes were illuminated with a dual wavelength fluorometer (CAM-230, Japan Spectroscopic, Tokyo, Japan). Video images were digitized, using an Argus-50/Ca system (Hamamatsu Photonics). The fluorescence ratio was calculated from the value of the fluorescence intensity at 500 nm with 340- and 380-nm excitation. The digital images were obtained every 5 min from just before treatment with CCCP till the end of the experiment.

2.3. Evaluation of morphological changes of cardiac myocytes

Cells lightly attached to the perfusion chamber were superfused with normal Tyrode solution at 37°C. After 30 min of stabilization, a rod-shaped cell was selected randomly from those within the microscopic field of the chamber. Caffeine (5 mM) was added for 1 min to the chamber in order to confirm that the function of the intracellular Ca^{2+} store of the myocyte was maintained. After washing out of the caffeine for 5 min, the morphology of the myocyte was recorded as a digital image using the Argus-50/Ca system. To examine the morphological change of the myocyte, the morphology of the same cell was also recorded at the end of chemical ischemia and also at the end of 30-min reperfusion. After Ca^{2+} measurement, the morphological change was analyzed by measuring the area of the myocyte. The cell surface area was calculated from the digitized image of the cell and the post-ischemic value was expressed as percentage of the pre-ischemic value.

2.4. Experimental protocols

In order to determine the optimal condition of the Ca^{2+} overload model by metabolic inhibition and energy repletion, we performed some preliminary experiments. We first followed a protocol for metabolic inhibition, using Tyrode solution containing normal Ca^{2+} and 1 μM of CCCP. However, the myocytes exposed to CCCP for 10–15 min showed a rigor contraction with an increase in $[Ca^{2+}]_i$, but failed to show Ca^{2+} overload on washout of CCCP (energy repletion) after the transition to rigor contraction (the sign of ATP depletion). These cells showed neither further $[Ca^{2+}]_i$ increase nor morphological changes after energy repletion. To improve the efficacy of the assessment of reperfusion injury, we used Ca^{2+}-free Tyrode solution for metabolic inhibition, since it was reported

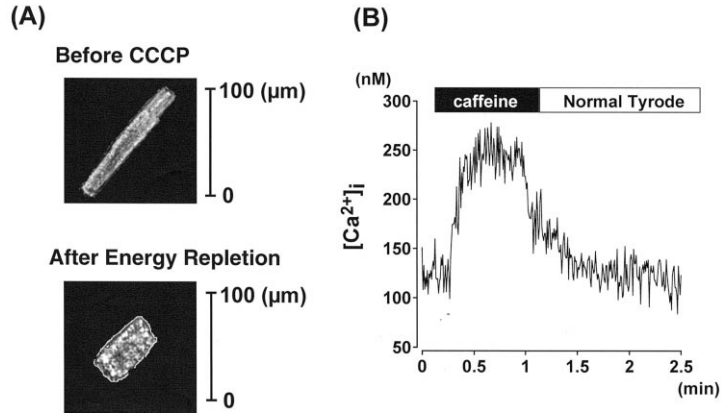

Fig. 1. (A) Typical morphology of a single cardiomyocyte. Before treatment with CCCP (1 μM), the myocytes were all rod-shaped, as shown in the upper panel. After perfusion with CCCP for 40 min followed by 30-min washout of CCCP (energy repletion), the myocytes became contracted (lower panel). (B) Typical effect of caffeine (5 mM) on the intracellular Ca^{2+} in the single fura-2-loaded guinea pig cardiomyocyte.

that cells in the low Ca^{2+} solution remain rod-shaped for a longer period against metabolic inhibition (Li et al., 1988). We also examined the dose-dependent effects of CCCP (100 nM, 1 and 3 μM), and confirmed that 1 μM of CCCP was the most stable to induce Ca^{2+} overload after energy repletion.

Based on these experiments, we established the standard procedure as follows. After confirmation of the integrity of the myocardial store functions described in the previous section, Ca^{2+}-free Tyrode containing CCCP (1 μM) was perfused for 40 min at a perfusion rate of 2–3 ml/min. Ca^{2+} overload was induced by washout of CCCP with normal Tyrode solution for 30 min at a perfusion rate of 4–5 ml/min. In this study, we employed the following two protocols: (A) before metabolic inhibition, in which drugs were included throughout the experiment, and (B) before energy repletion, in which drugs were included from 5 min before energy repletion to the end of the experiment.

2.5. Calibration for Ca^{2+} concentration

The maximum ratio (R_{max}) unique to individual cells was obtained at the end of each experiment by applying ionomycin (2 μM) to the normal Tyrode solution. The minimum ratio (R_{min}) was obtained by applying EGTA (10 mM) to the ionomycin containing Tyrode solution. Both R_{max} and R_{min} values were obtained under steady state conditions. $[Ca^{2+}]_i$ was calculated from the following formula: $[Ca^{2+}]_i\ (\mathrm{nM}) = (R - R_{min}) \times K_d \times F_{min}/((R_{max} - R) \times F_{max}))$, where R is the ratio obtained from the experiment, F means the fluorescence intensity at 380-nm excitation, and K_d is the dissociation constant defined as 224 nM.

2.6. Chemicals

L-*cis* Diltiazem and diltiazem were kind gifts from Tanabe Seiyaku (Osaka, Japan). A specific inhibitor of Na^+/Ca^{2+} exchanger, 2-[2-[4-(4-nitrobenzyloxy) phenyl] ethyl] isothiourea methanesulphonate (KB-R7943), was a kind gift from Kanebo (Osaka, Japan). CCCP was purchased from Sigma.

2.7. Statistical analysis

All data were expressed as means ± S.E.M. The time course data (Ca^{2+} measurement) were divided into two events, during 40 min of metabolic inhibition and during 30-min energy repletion, and repeated measures two-way analysis of variance was used. If there was no interaction between the drug factor and the time factor, Bonferroni/Dunn (Dunn's Procedure for Comparing a Control to

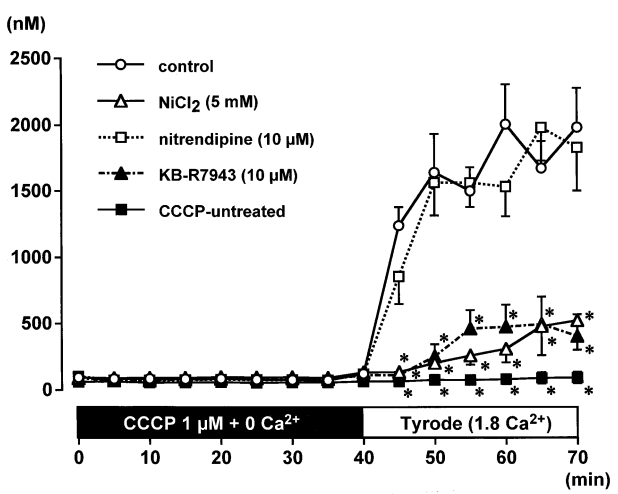

Fig. 2. Establishment of the model of Ca^{2+} overload induced by metabolic inhibition. Metabolic inhibition was induced by the perfusion of 1 μM CCCP in the Ca^{2+}-free medium and energy repletion was accomplished by switching the perfusate to normal Tyrode solution. Ni^{2+} or KB-R7943 was included 5 min before energy repletion until the end of the experiment, while nitrendipine was included throughout this experiment. As a negative control, the curve without CCCP is also shown. Each point represents the mean ± S.E.M. of three to nine experiments. $^*P < 0.01$ vs. control.

Table 1
Cell size
Effects of drugs on cell shortening and $[Ca^{2+}]_i$ increase induced by washout of CCCP. Values were obtained at the end of reperfusion. MI; drugs were included from the onset of metabolic inhibition to the end of the experiment, BR; drugs were included 5 min before energy repletion until the end of the experiment.

Treatment	Number	% Cell size	$[Ca^{2+}]_i$
Control	9	44±4	2000±290
Ni^{2+}	8	82±10[a]	520±220[b]
KB-R7943 (ER)	7	87±9[a]	500±230[b]
Nitrendine (MI)	8	50±5	1800±330
Nicardipine (MI)	3	48±11	1700±380
L-*cis* Diltiazem (ER)	8	91±7[a]	240±120[b]
Diltiazem 3 μM (ER)	8	65±9	1400±490
Diltiazem 10 μM (ER)	8	86±10[a]	200±50[b]

[a] $P < 0.05$.
[b] $P < 0.01$ vs. control.

All other Means) post-hoc test was done thereafter. One-way analysis of variance (ANOVA) followed by the Bonferroni/Dunn post-hoc test was used for the cell size data. All statistical analyses were performed using Super ANOVA™ (Abacus Concepts, Berkeley, CA). Differences were considered to be statistically significant when the P value was less than 0.05.

3. Results

3.1. Cell selection

In all the experiments, rod-shaped myocytes as shown in Fig. 1A were selected. A single myocyte was further selected for the ischemia experiment, based on the criterion that it showed a transient $[Ca^{2+}]_i$ increase on treatment with 5 mM caffeine (Fig. 1B). The cells, which did not satisfy these criteria, or which changed morphologically after the treatment with caffeine, were excluded.

3.2. Changes in $[Ca^{2+}]_i$ by metabolic inhibition and energy repletion

Fig. 2 represents the change in time course of $[Ca^{2+}]_i$ in the absence and the presence of drugs. Before CCCP treatment, the $[Ca^{2+}]_i$ of all cells used were almost identical in level (about 100 nM). In the control myocytes, $[Ca^{2+}]_i$ did not change during 40 min of CCCP treatment. After washout of CCCP, $[Ca^{2+}]_i$ increased dramatically within 5 min and continued to increase until the end of energy repletion. In order to confirm that this $[Ca^{2+}]_i$ increase was not due to the simple entry of extracellular Ca^{2+} when Ca^{2+}-free Tyrode was replaced by normal Tyrode, we performed the same perfusion protocol without CCCP treatment. We observed that the $[Ca^{2+}]_i$ showed a small (20–30 nM), but not significant, increase at 5 min after energy repletion. Therefore, the Ca^{2+} overload observed here is considered to be induced by the injury due to metabolic inhibition and energy repletion. Furthermore, Ni^{2+} (5 mM) and KB-R7943 (10 μM), known as inhibitors of the Na^+/Ca^{2+} exchanger, completely blocked the initial rise of $[Ca^{2+}]_i$ after washout of CCCP ($P < 0.01$ vs. control) and continued to attenuate the $[Ca^{2+}]_i$ increase during 30 min of energy repletion. However, nitrendipine (10 μM), a dihydropyridine Ca^{2+} channel blocker, failed to attenuate the $[Ca^{2+}]_i$ increase after washout of CCCP. Nicardipine (10 μM), another dihydropyridine Ca^{2+} channel blocker, also failed to attenuate the $[Ca^{2+}]_i$ increase (see Table 1). Thus, the $[Ca^{2+}]_i$ increase in this model was suggested to be mediated, not by L-type Ca^{2+} channels but by the Na^+/Ca^{2+} exchanger.

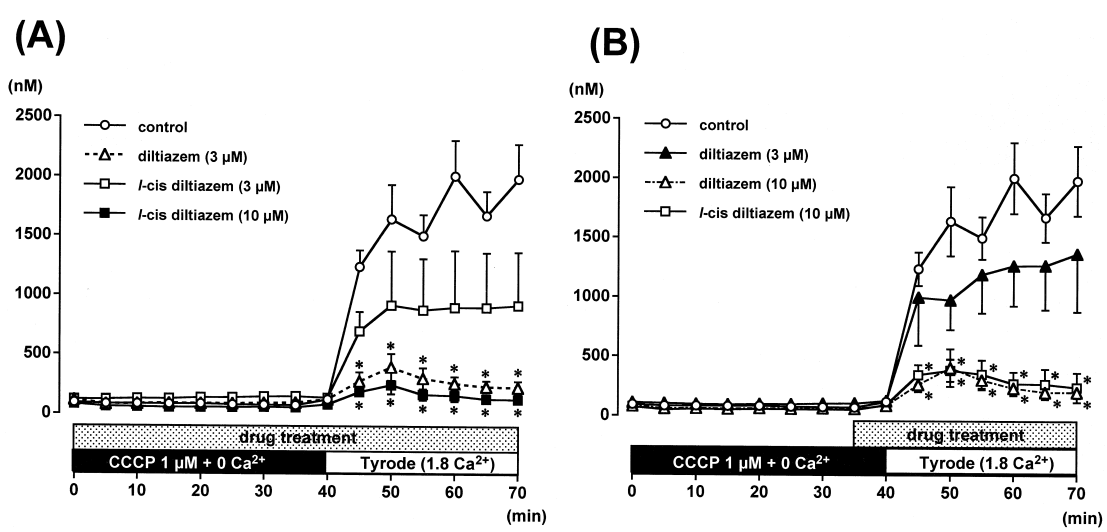

Fig. 3. (A) Effects of diltiazem and L-*cis* diltiazem on CCCP-induced Ca^{2+} overload. Drugs were included throughout the experiment. (B) Effects of diltiazem and L-*cis* diltiazem on Ca^{2+} overload when included 5 min before energy repletion until the end of the experiment. Control curves in A and B are the same data as that shown in Fig. 2. *$P < 0.01$ vs. control.

As for the treatment with drugs before metabolic inhibition (protocol (A)), diltiazem (3 μM) and L-*cis* diltiazem (10 μM), but not L-*cis* diltiazem (3 μM) significantly attenuated the $[Ca^{2+}]_i$ increase during energy repletion (Fig. 3A).

As for the treatment with drugs 5 min before washout of CCCP (protocol (B)), 3 μM of diltiazem, which was effective in protocol (A), failed to attenuate the $[Ca^{2+}]_i$ increase. However, a higher concentration of diltiazem (10 μM) could significantly attenuate the $[Ca^{2+}]_i$ increase after energy repletion (Fig. 3B). L-*cis* Diltiazem (10 μM) also attenuated the $[Ca^{2+}]_i$ increase ($P < 0.01$ vs. control).

3.3. Morphological change of the cell after energy repletion

Cells, which showed a rigor contraction during CCCP treatment, were all excluded from the experiments. Table 1 represents the morphological change in cell size caused by metabolic inhibition and energy repletion in the absence and the presence of drugs, accompanied by $[Ca^{2+}]_i$. Compared with the control, L-*cis* diltiazem (10 μM), diltiazem (10 μM), Ni^{2+} (5 mM) and KB-R7943 (10 μM), added 5 min before washout of CCCP, all reduced the cell shortening ($P < 0.05$), and these effects correlated well with the attenuation in $[Ca^{2+}]_i$ increase. Treatment with diltiazem (3 μM) failed to reduce the contraction, which correlated with the increase in $[Ca^{2+}]_i$. Neither nitrendipine (10 μM) nor nicardipine (10 μM) attenuated the contraction or the $[Ca^{2+}]_i$ increase after energy repletion.

4. Discussion

4.1. Increase in $[Ca^{2+}]_i$ by metabolic inhibition and energy repletion in guinea pig isolated myocytes

Isolated cardiomyocytes are reversibly made ATP-depleted by CCCP, a reversible inhibitor of mitochondrial oxidative phosphorylation (Li et al., 1989). The myocytes, which show a rigor contraction on metabolic inhibition are reported to have depressed Ca^{2+} overload, because the activity of the Na^+/Ca^{2+} exchanger is inhibited by energy depletion (Li et al., 1988; Collins et al., 1992). Li et al. (1988) have also reported that cells maintaining a low $[Ca^{2+}]_i$ level remain rod-shaped for a longer period against metabolic inhibition. Since our aim was to assess the effect of L-*cis* diltiazem on reperfusion-induced Ca^{2+} overload in vitro, we followed a protocol with Ca^{2+}-free Tyrode solution, which improves the efficacy of the assessment of reperfusion injury. Using this type of model, we could partially simulate the ischemia/reperfusion-induced Ca^{2+} overload in vitro by metabolic inhibition and energy repletion. A marked $[Ca^{2+}]_i$ increase was observed after washout of CCCP with Ca^{2+}-containing Tyrode solution. This $[Ca^{2+}]_i$ increase was suppressed by Ni^{2+} or KB-R7943, a specific inhibitor of the Na^+/Ca^{2+} exchanger, but was not suppressed by nitrendipine or nicardipine (Fig. 2). These results indicate that the $[Ca^{2+}]_i$ increase by energy repletion occurred via the Na^+/Ca^{2+} exchanger, but not through the dihydropyridine-sensitive Ca^{2+} channel in this model.

4.2. Cardioprotective effect of L-cis diltiazem on Ca^{2+} overload in guinea pig myocytes

In the present study, L-*cis* diltiazem and diltiazem attenuated both the $[Ca^{2+}]_i$ increase and the cell shortening induced by CCCP. Itogawa et al. (1996) demonstrated that both L-*cis* diltiazem and diltiazem attenuated veratridine-induced Ca^{2+} overload and hypercontracture in isolated rat hearts. However, this is not direct evidence that L-*cis* diltiazem actually protects against ischemia/reperfusion injury by inhibiting Ca^{2+} overload. In the present study, we mimicked the phenomenon of ischemia/reperfusion injury in the chamber and our data are the first demonstration that both L-*cis* diltiazem and diltiazem protect against the Ca^{2+} overload induced by metabolic inhibition and energy repletion in the same concentration range. This observation, together with the observation that nitrendipine and nicardipine were without effect, suggests that their effects are independent of L-type Ca^{2+} channel blocking. This possibility is also supported by the results of a previous study in our laboratory showing that L-*cis* diltiazem (10 μM) as well as diltiazem (3 μM) worked to preserve high-energy phosphates in the ischemia–reperfusion model of isolated guinea pig hearts (submitted for publication).

In the present study, inclusion of diltiazem (3 μM) throughout the experiment attenuated the $[Ca^{2+}]_i$ increase during reperfusion (Fig. 3A). However, the addition of diltiazem (3 μM), started 5 min before reperfusion, failed to attenuate the $[Ca^{2+}]_i$ increase (Fig. 3B). Thus, we suggest that some events during chemical ischemia, which could be affected by diltiazem, might be involved in the Ca^{2+} overload during reperfusion (Watts et al., 1990; Sakamoto et al., 1997).

4.3. Cardioprotective mechanism by L-cis diltiazem on Ca^{2+} overload by metabolic inhibition and energy repletion

In the present model, the $[Ca^{2+}]_i$ increase after energy repletion was inhibited by both Ni^{2+} and KB-R7943 (Fig. 2). Concerning the $[Ca^{2+}]_i$ increase in this model, the L-type Ca^{2+} channel does not seem to be involved. This is because the dihydropyridine Ca^{2+} channel blockers, nitrendipine and nicardipine, affected neither $[Ca^{2+}]_i$ increase nor cell shortening (Table 1). The inhibition of the Na^+/Ca^{2+} exchanger may afford protection from metabolic inhibition and energy repletion. A previous report has demonstrated that the intracellular Na^+ concentra-

tion ($[Na^+]_i$) is increased by metabolic inhibition in guinea pig myocytes (Satoh et al., 1995). This indicates that Na^+ overload caused during metabolic inhibition is important in this model. Itogawa et al. (1996) previously reported that L-*cis* diltiazem inhibited veratridine-induced Na^+ overload in rat cardiac myocytes. Haigney et al. (1992) reported that the $[Na^+]_i$ increase seen during hypoxia is potentiated after rigor contraction in isolated rat myocytes. They also suggested that inactivation-resistant Na^+ channels were involved in this $[Na^+]_i$ increase. We demonstrated in the present study that both L-*cis* diltiazem and diltiazem at 10 μM, when introduced 5 min before washout of CCCP, attenuated cell shortening after energy repletion (Fig. 3B). Thus, it is speculated that both L-*cis* diltiazem and diltiazem attenuate Ca^{2+} overload by inhibiting the $[Na^+]_i$ accumulation early after energy repletion.

In conclusion, we first demonstrated that L-*cis* diltiazem attenuated the Ca^{2+} overload caused by metabolic inhibition and energy repletion in guinea pig myocytes. The cardioprotective action of L-*cis* diltiazem evidenced in the present work merits further research, since L-*cis* diltiazem could become a lead compound for investigating protection against ischemia/reperfusion injury.

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