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Preservation of mitochondrial function during ischemia as a possible mechanism for cardioprotection of diltiazem against ischemia/reperfusion injury

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Abstract

A possible mechanism for D-*cis*-diltiazem (diltiazem)-mediated improvement of the contractile function of ischemic/reperfused hearts was examined. Thirty-five-min ischemia/60-min reperfusion recovered little the left ventricular developed pressure (LVDP) and decreased myocardial high-energy phosphates (HEPs). Ischemia induced an accumulation of tissue Na^+ content, an increase in cytochrome *c* in the cytosolic fraction, and a decrease in the oxygen consumption rate (OCR) in perfused hearts. Treatment of the heart with 1 μM diltiazem for the last 3-min of pre-ischemia did not affect the decrease in HEPs during ischemia, whereas that with 3 μM partially attenuated the decrease in ATP, suggesting that 3 μM diltiazem exerted energy-sparing effect. Treatment with 1 μM diltiazem enhanced the post-ischemic recovery of LVDP associated with attenuation of the ischemia-induced accumulation of tissue Na^+, increase in cytochrome *c* in the cytosolic fraction, and decrease in myocardial OCR, and restoration of the myocardial HEPs during reperfusion. Combined treatment with diltiazem and a Na^+/H^+ exchange inhibitor, but not a Na^+ channel blocker, facilitated the attenuation of Na^+ accumulation in the ischemic heart and the enhancement of the post-ischemic recovery of LVDP. Sodium lactate, a possible metabolite in ischemic hearts, and sodium chloride increased the Na^+ concentration in mitochondria, released cytochrome *c* into incubation medium, and reduced the mitochondrial respiration. Treatment of isolated mitochondria with diltiazem failed to attenuate the sodium lactate- and sodium chloride-induced alterations. These results suggest that the cardioprotection of diltiazem may be exerted via attenuating cytosolic Na^+ overload through Na^+ channels in the ischemic heart, leading to preservation of mitochondrial functional ability during ischemia, followed by improvement of post-ischemic energy production and contractile recovery.

Keywords: Diltiazem; Heart; Ischemia; Mitochondria; Na^+ channels; Na^+ overload

1. Introduction

There is accumulating evidence that diltiazem, a voltage-dependent Ca^{2+} channel blocker, protects cardiac function against ischemia/reperfusion- or hypoxia/reoxygenation-induced injury of the perfused heart [1–3]. Several possible mechanisms underlying the cardioprotective effects of diltiazem against ischemia/reperfusion injury has been considered including attenuation in Ca^{2+} overload [4] and energy-sparing effect due to negative inotropic effect [5]. Ca^{2+} overload during reperfusion is considered to occur due to enhancement of the reverse mode of Na^+/Ca^{2+} exchanger as a result of an accumulation of myocardial Na^+ content (Na^+ overload) during ischemia [6]. In addition, L-*cis*-diltiazem, which has at least 30-fold less potent in the Ca^{2+} channel blocking activity than D-*cis*-diltiazem (diltiazem) [7], exerted cardioprotection against ischemia/reperfusion injury [4,5,8]. Thus, blockade of L-type Ca^{2+} channels is unlikely attributable to cardioprotection of diltiazem against ischemia/reperfusion injury. Another possible mechanism underlying the cardioprotective effects against ischemia/reperfusion and hypoxia/reoxygenation injuries is an energy-sparing effect; that is, a better preservation of myocardial energy due to reduced contraction prior to or during ischemia may provide more utilizable energy for contraction during reperfusion [1,4,5,9]. However, substantial role of the

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Abbreviations: EIPA, ethylisopropyl amirolide; HEP, high-energy phosphate; LVDP, left ventricular developed pressure; OCR, oxygen consumption rate; TTX, tetrodotoxin.

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energy-sparing effects of diltiazem in the ischemia/reperfusion injury remains unclear.

Recently, we showed that post-ischemic contractile recovery of the perfused rat hearts was closely related to the mitochondrial function during ischemia [10] and that an attenuation of Na^+ accumulation in the ischemic myocardium by Na^+ channel blockers or Na^+/H^+ exchanger inhibitors may lead to protection of the mitochondrial function during ischemia as well as reperfusion, associated with improvement of post-ischemic recovery of contractile function of perfused hearts [3,11,12]. In the series of our studies [10–12], we suggested that Na^+ accumulation rather than Ca^{2+} accumulation during ischemia initiated mitochondrial damage, leading to the genesis of ischemia/reperfusion injury. In a previous study, we have shown that diltiazem attenuated ischemia-induced myocardial Na^+ overload [3]. However, it remains unclear how diltiazem attenuates Na^+ overload in the ischemic myocardium and how this drug affects mitochondria in the Na^+-overloaded myocardium. In the present study, we examined possible mechanisms by which diltiazem attenuates Na^+ accumulation and reduces subsequent damage to mitochondrial function in ischemic/reperfused rat hearts.

2. Materials and methods

2.1. Animal and agents

Male Wistar rats weighing 250–280 g (Japan Laboratory Animals Inc.) were used in the present study. The animals were conditioned to an environment of $23 \pm 1^\circ$, a constant humidity of $55 \pm 5\%$, and a cycle of 12-hr light/12-hr darkness and given free access to food and tap water according to the Guide for the Care and Use of Laboratory Animals as promulgated by the National Research Council (National Academy Press, Washington, DC, 1996). The protocol of this study was approved by the Committee of Animal Care and Welfare of Tokyo University of Pharmacy and Life Science.

The following agents were purchased: diltiazem and protease inhibitors (Wako), SBFI/AM (Mol. Probes), *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) and 2-(*N*-morpholino)-ethanesulfonate (MOPS) (Dojin), fatty acid-free BSA and Pluronic F-127 (Sigma).

2.2. Perfusion of isolated hearts

Perfusion of hearts and treatment with agents were performed according to the method described previously [11]. After anesthesia with diethyl ether, the hearts were rapidly isolated, transferred to the Langendorff apparatus, and perfused at 37° with a constant flow rate (9.0 mL/min) of the Krebs–Henseleit bicarbonate buffer of the following composition (mM): NaCl, 120; KCl, 4.8; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 1.25; $NaHCO_3$, 25; and glucose, 11 (pH 7.4). The perfusion buffer was equilibrated with a gas mixture of 95% O_2 + 5% CO_2. A latex balloon with an uninflated diameter of 3.7 mm was connected to a pressure transducer (TP-200, Nihonkohden), and inserted into the left ventricular cavity through the mitral opening to measure the LVDP. The heart was paced at 300 beats/min with an electrical stimulator via two silver electrodes directly attached to the heart and was subjected to 30-min equilibration. Hemodynamic parameters were recorded on a thermal pen recorder (WT-645G, Nihonkohden) throughout the experiment.

2.3. Ischemia/reperfusion and treatment with diltiazem

The perfusion was stopped for 35 min to induce global ischemia as described previously [3]. After 35-min ischemia, the hearts were reperfused for 60 min at 37° with the Krebs–Henseleit bicarbonate buffer. Treatment of perfused hearts with diltiazem was conducted by infusing the agent dissolved in the perfusion buffer into the portion just distal to the aortic cannula for the last 3-min prior to the start of ischemia as final concentrations of the agent ranging from 0.3 to 3 μM.

In the perfusion study, three series of perfusion experiments were performed. In the first series, hearts were subjected to 35-min ischemia or the following 60-min reperfusion with and without diltiazem treatment. After ischemia or reperfusion, content of myocardial HEP was determined. To determine whether diltiazem might exert an energy-sparing effect, myocardial HEPs were determined at 10 and 20 min ischemia, respectively. In the second series, hearts were also subjected to 35-min ischemia or the following 60-min reperfusion with and without diltiazem treatment and then their oxygen consumption rates were determined. In the third series of experiments, hearts were subjected to 35-min ischemia or the following 60-min reperfusion with and without treatment with 1 μM diltiazem, 0.3 μM TTX, 1 μM EIPA, or combination with two of these agents. This latter series was undertaken to investigate the route of Na^+ entry during ischemia. LVDPs of the perfused hearts treated with and without agents were determined at the end of reperfusion. Myocardial Na^+ content and oxygen consumption rate of skinned bundles were determined at the end of ischemia.

For the purpose of comparison, control hearts were perfused for 35 min or 95 min under normoxic conditions without any agent treatment.

2.4. Myocardial Na^+ and Ca^{2+} contents

Tissue Na^+ and Ca^{2+} contents were determined to assess the ionic disturbances in the heart. After pre-ischemia, ischemia, reperfusion, or normoxic perfusion, hearts were perfused for 1 min with 8.0 mL of ice-cold 320 mM sucrose–20 mM Tris–HCl, pH 7.4. Myocardial Na^+ and Ca^{2+} were extracted according to the method described

previously [3]. These ion concentrations were determined by means of an atomic absorption spectrometer (AA-680, Shimadzu).

In a previous study, we extensively characterized this method [3]. We found that cations determined by this method are devoid of those in the extracellular and vascular spaces of the heart.

2.5. Myocardial high-energy phosphates (HEPs)

After appropriate sequences of perfusion, hearts were clamped with aluminum tongs pre-cooled in liquid nitrogen and quickly frozen. The frozen tissue was pulverized in a mortar-driven homogenizer with a pestle and ATP and CP were extracted with 0.3 M perchloric acid containing 0.25 mM EDTA. The extracted ATP and CP were determined by means of HPLC (L2000 series, Hitachi) [10].

2.6. Oxygen consumption rate of perfused hearts

Myocardial OCR, a parameter for mitochondrial oxygen consumption of the perfused heart, was determined by the method described previously [13], a modified method of Saks *et al.* [14]. After appropriate sequences of perfusion, myocardial bundles, 0.3–0.4 mm in diameter and 3–4 mm in length, were prepared from the left ventricular free wall and the bundles were incubated in buffer for 20 min at 4° in the presence of 75 μg/mL saponin. After washout of saponin with the buffer, the OCR of skinned bundles was determined by means of a Clark-type electrode (Central Science). The basal OCR was measured in the absence of ADP and creatine. The total (maximal) OCR was measured in the presence of 1 mM ADP and 7.5 mM creatine. The ADP-stimulated OCR of the skinned bundles was taken as the difference between the maximal and basal glutamate/malate-stimulated OCRs.

2.7. Release of cytochrome c in ischemic hearts

At the end of ischemia, the heart was quickly removed from the perfusion apparatus and was homogenized with a glass-Teflon homogenizer at 600 rpm by one stroke for 1 min in an ice-cold buffer of the following composition (mM): mannitol, 210; sucrose, 70; DTT, 10; EDTA, 1 (pH 7.4). The buffer also included 2 μg/mL leupeptin, 2.5 μg/mL aprotonin, and 0.5 mM PMSF. The homogenate was centrifuged at 900 *g* for 10 min at 4°. The supernatant solution was centrifuged at 8000 *g* for 30 min at 4°. The resulting supernatant (cytosolic fraction) was centrifuged at 100,000 *g* for 30 min at 4° to remove any mitochondrial contamination and was used for Western blot. The cytosolic fraction was denatured in the Laemmli buffer at 100° and fractionated by SDS electrophoresis on a 15% polyacrylamide gel [15]. The fractionated proteins were then transferred onto a nitrocellulose filter, which was probed with a monoclonal anti-cytochrome *c* antibody (Santa Cruz Biotechnology), followed by horseradish peroxidase-conjugated donkey anti-mouse or rabbit IgG (Amersham Pharmacia Biotech). Bound antibody was detected by the chemiluminescence method (ECL®, Amersham Pharmacia Biotech).

2.8. Mitochondrial activity

2.8.1. Isolation of mitochondria

Cardiac mitochondria were prepared by the differential centrifugation method as described previously [10]. Mitochondria were resuspended with suspension buffer (320 mM sucrose, 10 mM Tris–HCl, pH 7.4) and used for measurement of mitochondrial activity. Protein concentrations were determined by the method of Lowry *et al.* [16].

2.8.2. Respiration of isolated mitochondria

Mitochondrial respiration activity was determined by the method described previously [10]. Isolated mitochondria (approximately 1 mg of protein) were placed in 1 mL of incubation medium containing 250 mM sucrose, 10 mM K_2HPO_4, and 10 mM glutamate (pH 7.4), and stirred at 30°. Mitochondrial oxidative phosphorylation was measured in the chamber using the Clark-type oxygen electrode as described above (Central Science). The quality of the mitochondrial preparation was determined using the respiratory control index (RCI) calculated as the ratio of the state 3 to the state 4 respirations.

2.8.3. Measurement of mitochondrial Na^+ concentration

Mitochondrial Na^+ concentration was determined by the method of Jung *et al.* [17] with slight modification. A membrane-permeable Na^+ indicator SBFI/AM was dissolved in DMSO at the concentration of 1 mM. Since SBFI/AM is a hydrophobic probe, 3-μL of 25% (w/v) Pluoronic F-127, a nonionic surfactant, was mixed with 3 μL of 1 mM SBFI/AM to enhance the loading the Na^+ indicator into the isolated mitochondria. The mixture was added to 200 μL of the incubation buffer of the following composition (mM): sucrose 250, EGTA 0.1, Tris–ATP 1.5, TES 5 (pH 7.4). The resultant solution was further mixed with 400-μL of the incubation medium containing isolated mitochondria and incubated for 30 min at 25°. After loading SBFI into mitochondria, the suspension was centrifuged at 8000 *g* at 25° for 5 min to remove the fluorescence probe in the extra-mitochondria.

SBFI-loaded mitochondria (600 μL suspension) in 1 mL cuvette were placed in fluorescence analyzer (CAF110, JASCO). Fluorescence of SBFI loaded in mitochondria was excited at 340 and 380 nm and emitted at 500 nm. Na^+ loaded in the mitochondria was expressed as the ratio of emission of fluorescence excited by the two-wavelengths [18].

The SBFI signals of isolated mitochondria were not affected by the presence of various concentrations of

choline chloride ranging from 6.25 to 50 mM under the present experimental conditions.

2.8.4. Release of cytochrome c from isolated mitochondria

Release of cytochrome *c* from the isolated mitochondria (about 1 mg protein) into 1 mL of incubation medium was determined according to the method described previously [12]. After incubation of the isolated mitochondria in the presence or absence of various concentrations of Na lactate or NaCl, the incubation medium was centrifuged at 10,000 *g* to remove isolated mitochondria. The resultant supernatant fluid was denatured in the Laemmli buffer at 100° and fractionated by SDS electrophoresis on a 15% polyacrylamide gel. Detection of the released cytochrome *c* was carried out, as described above.

2.9. Statistics

Each value represents the mean ± SEM. Statistical analyses were performed with the aid of StatView® for Windows (SAS Institute Inc.). Statistical significance was evaluated using analysis of variance (ANOVA) followed by Bonferroni's or Dunnett's multiple comparisons, if necessary. Differences with a probability of 5% or less were considered to be statistically significant.

3. Results

3.1. Contractile function of perfused hearts

Isolated rat hearts were subjected to 35-min ischemia and subsequent 60-min reperfusion with or without diltiazem treatment, and changes in LVDP were determined. Baseline (initial) values for LVDP of untreated and 0.3, 1, and 3 μM diltiazem-treated hearts were 80.0± 5.0 mmHg, 78.0 ± 2.5 mmHg, 76.0 ± 5.6 mmHg, and 82.1 ± 5.1 mmHg (N = 5 each), respectively. LVDPs of hearts treated without and with various concentrations of diltiazem prior to ischemia were 98.7 ± 1.3%, 86.0 ± 2.4%, 64.7 ± 4.7%, and 41.2 ± 4.7% of the corresponding baseline value, respectively. The ischemic/reperfused heart treated without agent showed a small LVDP (16.0 ± 3.3% of the initial value, N = 5) at the end of reperfusion. Treatment of perfused hearts with 0.3, 1, or 3 μM diltiazem resulted in an appreciable recovery of the LVDP after reperfusion (32.4 ± 3.5%, 68.5 ± 4.7%, and 90.9 ± 3.4%) of the corresponding baseline value, respectively. These results were similar to those in a previous study [3].

3.2. Myocardial Na^+ and Ca^{2+} contents

Myocardial Na^+ and Ca^{2+} contents were determined at the end of pre-ischemia, ischemia, or reperfusion. The baseline values for myocardial Na^+ and Ca^{2+} contents were 56.2 ± 1.2 μmol/g dry tissue and 1.87 ± 0.07 μmol/g dry tissue (N = 5), respectively. The myocardial Na^+ content at the end of ischemia increased to 120.0± 4.2 μmol/g dry tissue (N = 4). When the heart was subjected to 35-min ischemia and then reperfused, a further increase in myocardial Na^+ content was observed during reperfusion (147.5 ± 4.9 μmol/g dry tissue, N = 4). Myocardial Ca^{2+} content of the ischemic/reperfused heart increased to 13.14 ± 0.60 μmol/g dry tissue (N = 4), whereas the myocardial Ca^{2+} content did not change under the ischemic conditions (1.87 ± 0.09 μmol/g dry tissue, N = 4). Treatment with 0.3, 1, and 3 μM diltiazem attenuated the ischemia-induced increase in myocardial Na^+ content in a concentration-dependent manner (104.9± 3.6 μmol/g dry tissue, 84.5 ± 4.3 μmol/g dry tissue, and 75.7 ± 3.4 μmol/g dry tissue, respectively, N = 4 each). Treatment with diltiazem also attenuated the ischemia/reperfusion-induced increase in myocardial Na^+ content in a concentration-dependent manner (104.0 ± 4.0 μmol/g dry tissue, 78.8 ± 4.4 μmol/g dry tissue, and 67.7± 3.7 μmol/g dry tissue, respectively, N = 4 each). Treatment with diltiazem did not alter these cation contents prior to ischemia. Ischemia/reperfusion-induced increase in myocardial Ca^{2+} was also attenuated by treatment with diltiazem (10.46 ± 0.63 μmol/g dry tissue, 5.90 ± 0.66 μmol/g dry tissue, and 3.34 ± 0.40 μmol/g dry tissue, respectively, N = 4 each). These results were similar to those in a previous study [3].

3.3. Myocardial high-energy phosphates (HEPs)

ATP and CP contents of the untreated heart at the end of the pre-ischemia were 24.4 ± 0.6 μmol/g dry tissue and 34.9 ± 0.6 μmol/g dry tissue, respectively (N = 5). Myocardial ATP content of the untreated heart was decreased during ischemia, whereas myocardial CP content of the untreated heart was rapidly decreased (Fig. 1).

When hearts were treated with 0.3 or 1 μM diltiazem, changes in ATP and CP contents at any ischemic period examined were similar to those of the untreated heart. In contrast, the ATP content of the heart treated with 3 μM diltiazem at 10 or 20 min of ischemia was higher than that of the untreated heart, whereas ATP content at 35 min of ischemia was similar to that of the untreated heart (upper panel in Fig. 1). CP content of the heart treated with diltiazem was similar to that of the untreated heart at any ischemic period examined (lower panel in Fig. 1). When the diltiazem-treated hearts were reperfused, myocardial ATP and CP contents were restored in a concentration-dependent manner.

3.4. Myocardial oxygen consumption rate

The ADP-stimulated OCR of the left ventricular skinned bundles of the control heart prior to ischemia and of the

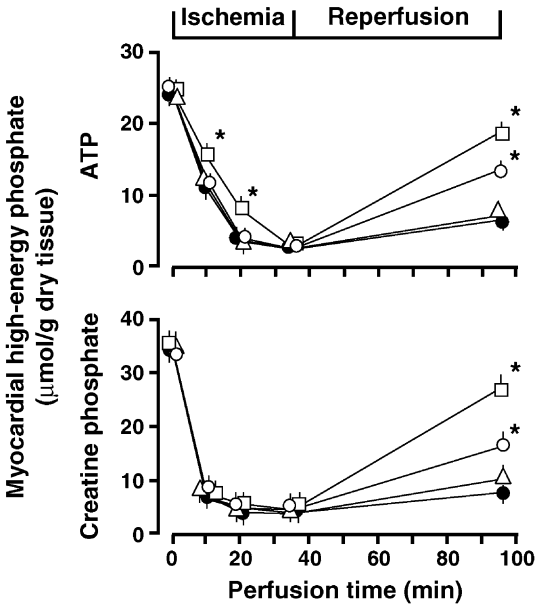

Fig. 1. The time course of changes in ATP (upper panel) and creatine phosphate (CP) contents (lower panel) of perfused hearts untreated (closed circles) or treated with 0.3 (open triangles), 1 μM (open circles), or 3 μM (open squares) diltiazem during ischemia/reperfusion, respectively. Each value represents the mean ± SEM of four or five experiments. (∗) Significantly different from the corresponding untreated groups ($P < 0.05$).

diltiazem-treated heart at the end of ischemia or reperfusion was determined (Fig. 2). The OCR for the pre-ischemic heart was 63.87 ± 0.91 nanoatom O/min/mg protein (N = 5). There were no significant differences in the OCR between diltiazem-treated and untreated, normoxic hearts. The OCR of the untreated heart under ischemic conditions was significantly reduced to approximately 40% of the value for the normoxic heart (N = 4). The OCR of the reperfused heart was further reduced to approximately 30% of the value for the normoxic heart

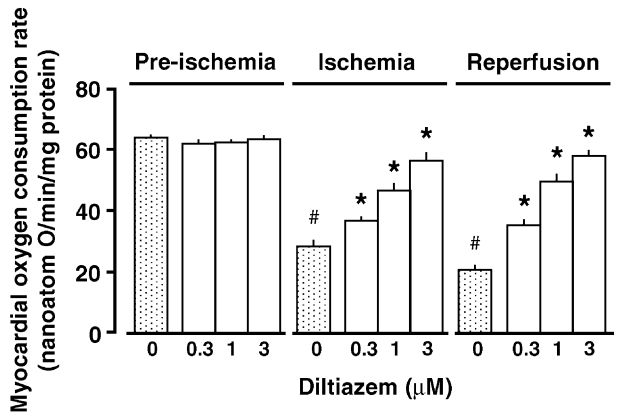

Fig. 2. Oxygen consumption rate (ADP-dependent minus basal oxygen consumption rate) of left ventricular skinned bundles prepared from perfused hearts untreated (0 μM diltiazem) or treated with 0.3, 1, or 3 μM diltiazem at the ends of pre-ischemia, ischemia, and reperfusion, respectively. Each value represents the mean ± SEM of five experiments. (#) Significantly different from the untreated, pre-ischemic group (0 μM diltiazem of "Pre-ischemia"; $P < 0.05$). (∗) Significantly different from the corresponding untreated groups ($P < 0.05$).

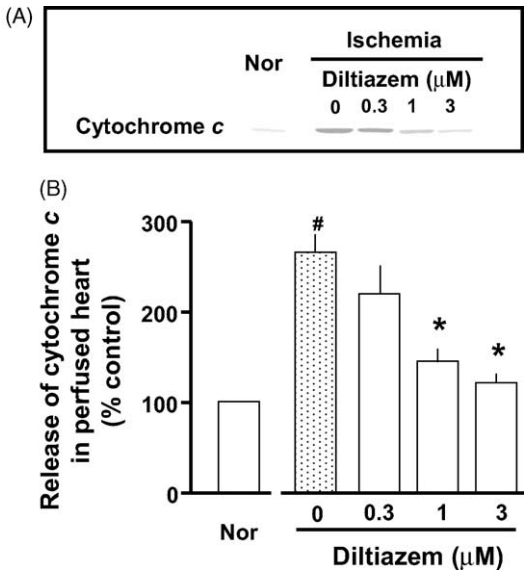

Fig. 3. Representative Western blots of cytochrome *c* released from the mitochondria (A) and effects of treatment without (0 μM diltiazem) or with 0.3, 1, or 3 μM diltiazem on the release of cytochrome *c* from the mitochondria into the cytosol in the ischemic heart (Ischemia), respectively (B). Each value is expressed as the percentage of the heart without ischemia/reperfusion and diltiazem treatment (the control heart; Nor) and represents the mean ± SEM of five experiments. Very faint band of cytochrome *c* for normoxia was detected. (#) Significantly different from Nor group ($P < 0.05$). (∗) Significantly different from the corresponding untreated ischemic group (0 μM; $P < 0.05$).

(N = 5). In contrast, treatment with diltiazem concentration-dependently preserved the OCR at the ends of both ischemia and reperfusion (N = 5 each).

3.5. Release of cytochrome c in ischemic hearts

The cytosolic fraction from the perfused heart after 35-min ischemia was prepared to determine cytochrome *c* release from the mitochondria (N = 5 each, Fig. 3). The amount of cytochrome *c* released from the mitochondria in the normoxic heart was very low. When the heart was subjected to ischemia, a marked increase in cytochrome *c* in the cytosolic fraction was seen at the end of ischemia. The increase in the release of cytochrome *c* was concentration-dependently attenuated by treatment with diltiazem.

Cytochrome *c* oxidase, a marker of mitochondria, was not detected by the Western blotting method in the resultant supernatant fluids prepared from normoxic and ischemic myocardium. The results suggested that cytochrome *c* detected in the present study was released from mitochondria and that the fraction unlikely contained fragmented mitochondria.

3.6. Effects of combined treatment of diltiazem with the Na^+ channel blocker or the Na^+/H^+ exchange inhibitor

Post-ischemic LVDP of the heart treated with 1 μM diltiazem, 0.3 μM TTX, or 1 μM EIPA alone or combinations of two of these agents is shown in Table 1. Treatment

Table 1
The recovery of left ventricular developed pressure (LVDP) of the ischemic/reperfused heart untreated (Un) and treated with 1 μM diltiazem (Dil), 0.3 μM tetrodotoxin (TTX), 1 μM ethylisopropyl amirolide (EIPA), or a combination of 1 μM Dil and 0.3 μM TTX (Dil + TTX), 1 μM Dil and 1 μM EIPA (Dil + EIPA), or 0.3 μM TTX and 1 μM EIPA (TTX + EIPA)

Treatment	Initial (mmHg)	At the ends of normoxia or ischemia/reperfusion (mmHg)
Normoxia	81.6 ± 3.3	78.6 ± 6.8
Un	84.2 ± 4.1	16.0 ± 3.3[#]
Agent alone		
Dil	79.9 ± 4.8	52.0 ± 5.1[*]
TTX	83.3 ± 5.1	48.3 ± 3.6[*]
EIPA	86.7 ± 5.8	55.3 ± 4.2[*]
Combined		
Dil + TTX	80.1 ± 3.8	54.1 ± 3.8[*]
Dil + EIPA	78.1 ± 3.9	72.2 ± 6.8[*,†]
TTX + EIPA	77.2 ± 4.2	71.3 ± 4.1[*,†]

Treatment with Dil and TTX was conducted for the last 3-min of pre-ischemia, and that with EIPA for the last 15-min. The group of the normoxic heart is shown as Normoxia. Each value represents the mean ± SEM of four or five experiments.

[#]Significantly different from the untreated, normoxic group (Normoxia; $P < 0.05$).

[*]Significantly different from the untreated group ($P < 0.05$).

[†]Additional effects of combinations of the two groups were obtained ($P < 0.05$).

of the perfused heart with diltiazem, TTX, or EIPA resulted in approximately 60–65% of post-ischemic LVDP recovery of the initial value. Treatment of the heart with combination of diltiazem with EIPA or of TTX with EIPA resulted in more than 90% recovery of the LVDP. In contrast, treatment of the heart with both diltiazem and TTX recovered approximately 60% of the initial value after reperfusion.

Myocardial Na^+ content at the end of ischemia is shown at the upper panel in Fig. 4. The Na^+ content increased to approximately 2-fold the baseline value at the end of ischemia. At 35 min ischemia, the Na^+ content of the heart treated with diltiazem, TTX, or EIPA alone was approximately 85 μmol/g dry tissue (N = 4 each). Treatment with the combination of diltiazem with EIPA or of TTX with EIPA reduced the myocardial Na^+ content to approximately 65 μmol/g dry tissue (N = 4 each), whereas

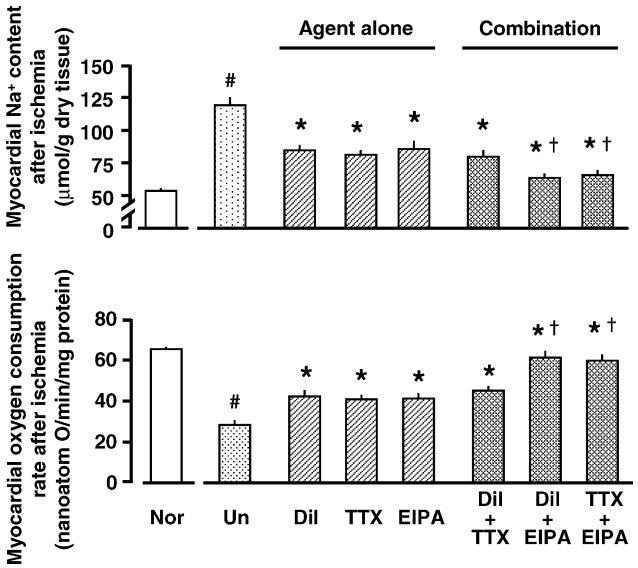

Fig. 4. Na^+ content of the ischemic heart (the upper panel), and oxygen consumption rate (OCR) of the skinned bundles prepared from the ischemic heart (the lower panel) untreated (Un) and treated with 1 μM diltiazem (Dil), 0.3 μM tetrodotoxin (TTX), 1 μM ethylisopropyl amirolide (EIPA), or a combination of 1 μM diltiazem and 0.3 μM TTX (Dil + TTX), 1 μM diltiazem and 1 μM EIPA (Dil + EIPA), or 0.3 μM TTX and 1 μM EIPA (TTX + EIPA). Treatment with diltiazem and TTX was conducted for the last 3-min of pre-ischemia, and that with EIPA for the last 15-min. The group of the normoxic heart is shown as Nor. Each value represents the mean ± SEM of four or five experiments. (#) Significantly different from the untreated, normoxic group (Nor; $P < 0.05$). (∗) Significantly different from the untreated group ($P < 0.05$). (†) Additional effects of combinations of the two groups were obtained ($P < 0.05$).

the Na^+ content of the heart treated with combination of diltiazem with TTX was similar to that with diltiazem or TTX alone.

The OCR of skinned bundles prepared from the ischemic myocardium with and without agent treatment is shown at the lower panel in Fig. 4. Treatment of the perfused hearts with TTX or EIPA attenuated the decrease in myocardial OCR: the OCR value was similar to that for the heart treated with 1 μM diltiazem. Treatment with the combination of diltiazem with EIPA or of TTX with EIPA attenuated the decrease in the OCR in the ischemic myocardium (N = 4 each), whereas the OCR of the heart

Table 2
Effects of NaCl and Na lactate on the respiratory parameters of isolated mitochondria

	Control	In the presence of NaCl (mM)				In the presence of Na lactate (mM)			
		12.5	25	50	100	12.5	25	50	100
State 3	282 ± 15	253 ± 14[*]	225 ± 7[*]	179 ± 11[*]	151 ± 12[*]	234 ± 11[*]	175 ± 6[*]	109 ± 9[*]	94 ± 4[*]
State 4	17.5 ± 0.9	23.1 ± 1.6[*]	25.1 ± 1.1[*]	30.8 ± 1.7[*]	36.2 ± 2.4[*]	36.5 ± 0.9[*]	54.2 ± 2.0[*]	69.9 ± 2.2[*]	80.0 ± 2.9[*]
RCI	16.2 ± 0.5	11.2 ± 0.6[*]	9.1 ± 0.6[*]	5.8 ± 0.4[*]	4.2 ± 0.1[*]	6.7 ± 1.0[*]	3.2 ± 0.1[*]	1.6 ± 0.2[*]	1.2 ± 0.1[*]
ADP/O	2.64 ± 0.05	2.45 ± 0.06[*]	2.20 ± 0.07[*]	1.92 ± 0.08[*]	1.75 ± 0.09[*]	2.25 ± 0.06[*]	1.94 ± 0.09[*]	1.58 ± 0.06[*]	1.38 ± 0.05[*]
OPR	746 ± 44	620 ± 39[*]	495 ± 15[*]	345 ± 32[*]	267 ± 32[*]	526 ± 20[*]	339 ± 19[*]	172 ± 19[*]	130 ± 6[*]

Each value represents the mean ± SEM of four or five measurements. Abbreviations: State 3, state 3 respiration (nanoatom O/min/mg protein); State 4, state 4 respiration (nanoatom O/min/mg protein); RCI, respiratory control index; ADP/O, ratio of ADP and oxygen atom, OPR; oxidative phosphorylation rate (nanoatom ADP/min/mg protein).

[*]Significantly different from the control group ($P < 0.05$).

Table 3
Effects of diltiazem on the respiratory parameters of isolated mitochondria in the presence and absence of 25 mM NaCl or 25 mM Na lactate

	In the presence of 25 mM NaCl					In the presence of 25 mM Na lactate				
	Diltiazem (μM)					Diltiazem (μM)				
	0	0.3	1	3	10	0	0.3	1	3	10
State 3	225 ± 7	224 ± 12	214 ± 15	227 ± 9	219 ± 12	175 ± 6	169 ± 7	178 ± 10	176 ± 12	174 ± 10
State 4	25.1 ± 1.1	25.9 ± 1.7	25.0 ± 0.8	25.5 ± 0.9	26.0 ± 1.0	54.2 ± 2.0	53.7 ± 1.1	54.2 ± 1.4	54.1 ± 1.2	54.6 ± 1.0
RCI	9.1 ± 0.6	8.7 ± 0.3	8.7 ± 0.9	8.9 ± 0.5	8.6 ± 0.7	3.2 ± 0.1	3.2 ± 0.1	3.3 ± 0.2	3.3 ± 0.3	3.3 ± 0.2
ADP/O	2.20 ± 0.07	2.20 ± 0.07	2.20 ± 0.11	2.10 ± 0.08	2.18 ± 0.05	1.94 ± 0.09	1.93 ± 0.09	1.80 ± 0.09	1.90 ± 0.09	1.93 ± 0.10
OPR	495 ± 15	494 ± 35	474 ± 53	477 ± 32	483 ± 21	339 ± 19	324 ± 9	320 ± 17	333 ± 29	344 ± 40

Each value represents the mean ± SEM of four or five measurements. There were no significant differences in the mitochondrial parameters among the groups treated with and without various concentrations of diltiazem ranging from 0.3 to 10 μM in the presence of 25 mM NaCl or 25 mM Na lactate. Abbreviations: State 3, state 3 respiration (nanoatom O/min/mg protein); State 4, state 4 respiration (nanoaotm O/min/mg protein); RCI, respiratory control index; ADP/O, ratio of ADP and oxygen atom, OPR; oxidative phosphorylation rate (nanoatom ADP/min/mg protein).

treated with combination of diltiazem with TTX was similar to that with diltiazem or TTX alone.

3.7. Respiration of isolated mitochondria under Na^+-loaded conditions

To examine the effects of Na^+ on the mitochondrial activity *in vitro*, the state 3 and 4 respirations, respiratory control index, ADP/O ratio, and oxidative phosphorylation rate of the isolated mitochondria were determined in the presence and absence of different concentrations of NaCl or Na lactate (Table 2). These parameters for mitochondrial respiration were decreased with increased concentration of NaCl or Na lactate (Table 2). The decrease in the parameters by Na lactate was greater than that by NaCl. Diltiazem at the concentrations of 0.3–10 μM failed to attenuate the 25 mM NaCl- or 25 mM Na lactate-induced decrease in respiratory parameters of isolated mitochondria (Table 3). When the mitochondria were incubated in the presence of diltiazem under NaCl- or Na lactate-free conditions, the value for the respiration was similar to that for diltiazem-untreated mitochondria (data not shown).

3.8. Na^+ entry in mitochondria

Figure 5 shows changes in the SBFI signal of isolated mitochondria in the presence of various concentrations of NaCl or Na lactate and effects of diltiazem, TTX, or EIPA on Na lactate-induced increase in SBFI signal. The SBFI signal of the mitochondria was increased concentration-dependently by the presence of NaCl (left upper panel in Fig. 5). When mitochondria were incubated in the medium including various concentrations of Na lactate instead of NaCl, the increase in the SBFI signal of the mitochondria was greater than that induced by NaCl.

Diltiazem at various concentrations ranging from 0.3 to 10 μM did not affect the baseline value for the SBFI signal. The NaCl- or Na lactate-induced increase in the mitochondrial SBFI signal was not affected regardless of the presence or absence of 0.3–10 μM diltiazem (right upper panel in Fig. 5). Incubation of isolated mitochondria with TTX (0.1–1 μM) or EIPA (0.3–3 μM) did not affect the NaCl- or Na lactate-induced increase in the mitochondrial SBFI signal (lower panel in Fig. 5).

3.9. Release of cytochrome c from isolated mitochondria

To determine the effect of diltiazem on NaCl- and Na lactate-induced release of cytochrome *c* from the mitochondria *in vitro*, the isolated mitochondria were incubated

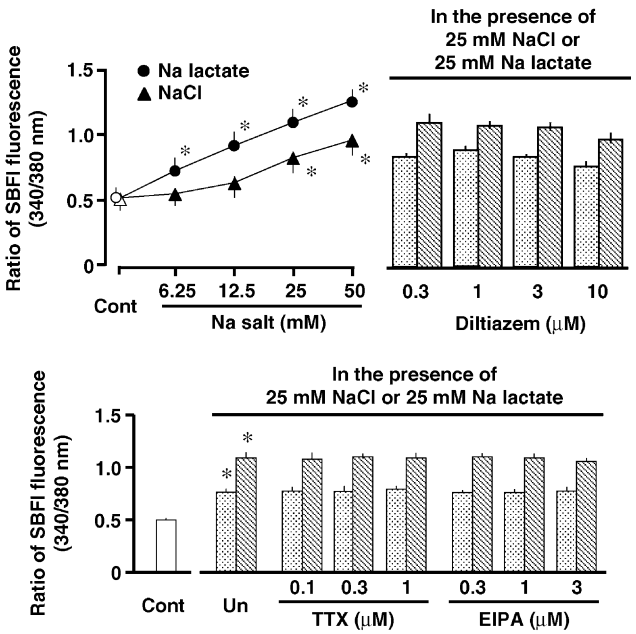

Fig. 5. Changes in fluorescence ratio of the SBFI-loaded mitochondria in the absence (Cont; open symbols) and the presence of various concentrations of NaCl (closed triangles) or Na lactate (closed circles) (the left upper panel), and effects of 0.3–10 μM diltiazem on the NaCl-induced (hatched columns) or Na lactate-induced increase (striped columns) in the SBFI ratio of isolated mitochondria (the right upper panel). The lower panel shows the effects of tetrodotoxin (TTX), or ethylisopropyl amirolide (EIPA) on NaCl- or Na lactate-induced changes in SBFI ratio of isolated mitochondria. Each value represents the mean ± SEM of five experiments. (*) Significantly different from the group in the absence of sodium salts ($P < 0.05$).

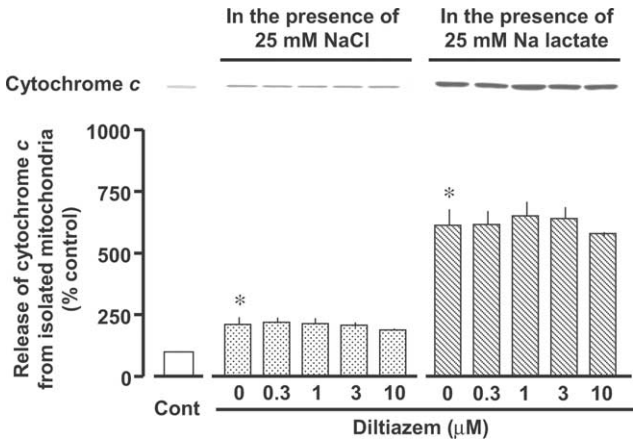

Fig. 6. Representative Western blots of cytochrome *c* released from the isolated mitochondria into incubation medium (upper panel) and the effect of diltiazem on 25 mM NaCl-induced (hatched columns) or Na lactate (striped columns)-induced release of cytochrome *c* from the isolated mitochondria (lower panel). Each value is expressed as the percentage of released amount of cytochrome *c* for the mitochondria incubated without 25 mM Na salts (Cont) and represents the mean ± SEM of five measurements. Very faint band of cytochrome *c* for control was detected. There were no significant differences in the released amount of cytochrome *c* among experimental groups in the presence of Na salts.

with diltiazem in the presence of 25 mM NaCl or 25 mM Na lactate (Fig. 6). The supernatant solution of the incubation medium was collected and subjected to Western blot analysis with a monoclonal antibody against cytochrome *c*. In the absence of sodium salts, the release of cytochrome *c* was very low. In contrast, appreciable amounts of cytochrome *c* were released from the isolated mitochondria during 30 min incubation in the presence of Na lactate. The release of cytochrome *c* from the mitochondria was also seen in the presence of 25 mM NaCl, but to a lesser degree than 25 mM Na lactate. Treatment with diltiazem at different concentrations failed to attenuate the Na lactate-induced release of cytochrome *c* from the mitochondria.

4. Discussion

As described in a previous study [3], treatment of perfused hearts with diltiazem enhanced post-ischemic contractile recovery. In the present study, we confirmed that diltiazem-mediated improvement of contractile recovery of ischemic/reperfused hearts was associated with reperfusion-evoked restoration of myocardial HEPs, suggesting that diltiazem is capable of exerting functional and metabolic cardioprotection of ischemic/reperfused hearts, which is in good agreement with those previously reported by others [1,9].

Several possible mechanisms underlying the cardioprotection of diltiazem against ischemia/reperfusion injury have been postulated. It is generally considered that agents that have negative inotropic effects may exert their cardioprotective action through energy-sparing effects [5,9]. A significant negative inotropic effect of diltiazem on the perfused heart during the injection of the drug in a concentration-dependent manner was detected [3]. In the present study, we observed higher ATP levels of the 3 μM diltiazem-treated heart at 10 or 20 min of ischemia than that of the untreated heart. In contrast, there were no significant differences in myocardial ATP content between the 1 μM diltiazem-treated and untreated groups at any ischemic period examined. These results suggest that 3 μM, but not 1 μM, diltiazem may exert an energy-sparing effect to some extent during ischemia. Since post-ischemic recovery of LVDP of the heart treated with 3 μM diltiazem (approximately 90% of the initial value) was greater than that with 1 μM diltiazem (approximately 65%), an energy-sparing effect may contribute to the better post-ischemic recovery of myocardial contractile function.

The post-ischemic LVDP recovered only 16% of the initial value in the diltiazem-untreated heart, whereas it recovered to 65% of the initial by treatment with 1 μM diltiazem that did not exert an energy-sparing effect during ischemia. The finding suggests that another mechanism underlying the cardioprotection of diltiazem against ischemia/reperfusion injury, which differs from energy-sparing effect, must be considered.

In the present study, only Na^+ content was accumulated during ischemia and Na^+ and Ca^{2+} contents during reperfusion, suggesting that non-specific accumulation in myocardial cations during ischemia is unlikely. The NMR study detected 2- to 4-fold increase in the intracellular free Ca^{2+} after 20–30 min ischemia [19]. Inoue *et al.* showed that epicardial, free Ca^{2+} concentration of the perfused heart was increased to a small but appreciable degree during the ischemia as detected by Fura 2-fluorescence probe [20]. However, we did not find any significant increase in myocardial Ca^{2+} at the end of ischemia. The different profiles in the Ca^{2+} concentration may be due to the ability of the methods to determine cations: detectable Ca^{2+} concentrations by the NMR or Ca^{2+} fluorescence probe method may range from nM to μM, whereas those by the atomic absorption method, μM to mM. Despite such difference, our findings showed that ischemia-induced ionic disturbance was initiated by the massive influx of Na^+ during ischemia, whereas the massive Ca^{2+} accumulation was unlikely occurred during ischemia. The findings motivated us to focus on the role of Na^+ accumulation in the ischemic myocardium in the ischemia/reperfusion injury.

In a previous study, we reported that myocardial Na^+ content was accumulated during ischemia and that diltiazem attenuated ischemia-induced Na^+ accumulation [3]. It is generally considered that Na^+ accumulation in the ischemic myocardium may be induced via Na^+ channels and Na^+/H^+ exchanger [21]. In fact, Na^+ channel blockers and Na^+/H^+ exchanger inhibitors attenuated ischemia- or ischemia/reperfusion-induced Na^+ accumulation associated with a better recovery of contractile function of

the perfused heart [3,11,12]. However, it is unclear which pathway of Na^+ influx may be affected by diltiazem. To investigate the route of Na^+ entry during ischemia, we performed a combined study using diltiazem, a Na^+ channel blocker TTX, and a Na^+/H^+ exchange inhibitor EIPA in the ischemic/reperfused hearts under the same experimental conditions. As shown in previous reports, the heart treated with 1 μM diltiazem [3], 0.3 μM TTX [21] or 1 μM EIPA [12] showed a similar degree of myocardial Na^+ content at the end of ischemia as well as a similar degree of post-ischemic contractile recovery. Combination of diltiazem with EIPA, or of TTX with EIPA attenuated the ischemia-induced Na^+ accumulation and enhanced post-ischemic LVDP recovery to a greater extent than each agent alone. In contrast, the combination of diltiazem with TTX elicited neither an additional reduction in the ischemia-induced Na^+ accumulation nor an additional recovery of post-ischemic LVDP compared with diltiazem or TTX alone. These findings suggest that the pathway of Na^+ influx attenuated by diltiazem may be similar to that by TTX, that is, Na^+ influx through Na^+ channels, but different from that by EIPA.

Recently, Ju *et al.* reported that there was a Na^+ current that operated during hypoxia, which could be blocked by TTX and lidocaine [22]. However, no information is available concerning the effect of diltiazem on this Na^+ current under ischemic conditions, and this Na^+ current in the ischemic myocardium has not yet been clarified. The electrophysiological study of Guc *et al.* showed that 5 μM diltiazem reduced myocardial Na^+ channel activity under normoxic conditions [23]. Although the concentration of diltiazem that was employed in their study was relatively higher than that in the present study, the present results suggest that 1 μM diltiazem might exert attenuation in myocardial Na^+ influx under pathophysiological conditions. Taken together, it is likely that diltiazem inhibits the ischemia-induced Na^+ influx probably through Na^+ channels, leading to the cardioprotection against ischemia/reperfusion injury of the perfused rat heart.

Question arises as to how an increase in cytosolic Na^+ may work in the ischemic/reperfused heart. We found in the *in vitro* study an increase in the mitochondrial Na^+ concentration in the presence of NaCl or Na lactate. We also found greater degree of Na^+ accumulation in mitochondria by Na lactate, a possible metabolite in ischemia, than that by NaCl. Although it remains unclear what kind of pathway contributes to Na^+ influx to mitochondrial matrix, our results suggest that lactate accumulated in the ischemic myocardium may enhance Na^+ flux rate into mitochondria.

Furthermore, the *in vitro* study using isolated mitochondria showed that NaCl and Na lactate induced a decrease in the mitochondrial respiration from the isolated mitochondria. These results suggest that cytosolic Na^+ overload may cause the mitochondrial damage during ischemia, which may be exerted via Na^+ influx from cytosol into mitochondria (cytosolic Na^+ overload-induced mitochondrial Na^+ overload). At the end of ischemia, cytochrome *c* was released from the mitochondria into the cytosolic fraction in the perfused heart. Boutaite *et al.* also showed a decrease in mitochondrial cytochrome *c* and a release of cytochrome *c* into cytosol in the ischemic myocardium [24]. They also suggested that supplement of cytochrome *c* into mitochondria might reverse ischemia-induced decrease in mitochondrial function. It is generally recognized that release of cytochrome *c* is a marker of mitochondrial damage [24] or of opening of membrane permeability transition (MPT) pore [25,26]. Furthermore, we have shown in previous studies that Na^+ caused mitochondrial swelling and a decrease in mitochondrial membrane potential [10,11]. Therefore, it is likely that attenuation of Na^+ influx to ischemic myocardium may play an important role for cardiac protection against ischemia/reperfusion injury. Interestingly, the effects of NaCl or Na lactate on the oxidative phosphorylation and cytochrome *c* release of the isolated mitochondria were not cancelled by the presence of diltiazem *in vitro*. In the present study, we have shown that Na^+ accumulation, that is, mitochondrial Na^+ overload was not prevented by not only diltiazem but also TTX, or EIPA. These findings suggest that the beneficial effects of diltiazem do not affect the mitochondria directly, but were mediated through modification of Na^+ accumulation in cytosol during ischemia. Clearly, preservation of the mitochondrial function during ischemia can retain the ability of mitochondria to generate HEPs, resulting in a restoration of myocardial HEPs in the reperfused heart. In accordance with this, the present findings showed that the myocardial OCR, a marker of mitochondrial ability to produce HEPs, was preserved during ischemia and reversed during reperfusion in the diltiazem-treated hearts associated with better restoration of HEPs and contractile function.

In conclusion, we examined the cardioprotective effect of diltiazem on the ischemic/reperfused hearts. Ischemia induces a massive increase in myocardial Na^+ content, which may lead to deterioration of the mitochondrial ability to produce ATP during ischemia, that is, cytosolic Na^+ overload-induced mitochondrial Na^+ overload may induce mitochondrial dysfunction, possibly through opening of mitochondrial permeability transition pore. This disability may last during reperfusion and cause an impairment of energy production, eventually leading to contractile failure of the reperfused heart. The mechanism by which diltiazem exerts a cardioprotective effect may be attributed to the preservation of mitochondrial function during ischemia probably by the blockade of ischemia-induced Na^+ influx through Na^+ channels.

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