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Both D-*cis*- and L-*cis*-diltiazem attenuate hydrogen peroxide-induced derangements in rat hearts

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Abstract

The effects of D-*cis*- and L-*cis*-diltiazem on the hydrogen peroxide (H_2O_2)-induced derangements of mechanical function and energy metabolism, and accumulation of intracellular Na^+ were studied in isolated rat hearts. The intracellular concentration of Na^+ ($[Na^+]_i$) in the myocardium was measured using a nuclear magnetic resonance technique. H_2O_2 (600 μM) increased the left ventricular end-diastolic pressure, decreased the tissue level of ATP, and increased the release of lactate dehydrogenase from the myocardium. These alterations induced by H_2O_2 were significantly attenuated by D-*cis*-diltiazem (15 μM) or L-*cis*-diltiazem (15 μM). H_2O_2 (1 mM) produced a marked increase in the myocardial $[Na^+]_i$, which was effectively inhibited by tetrodotoxin (3 μM), D-*cis*-diltiazem (15 μM) or L-*cis*-diltiazem (15 μM). These results suggest that both D-*cis*- and L-*cis*-diltiazem protect the myocardium against the H_2O_2-induced derangements in the isolated, perfused rat heart. The protective action of D-*cis*- and L-*cis*-diltiazem may be due to their ability to inhibit the H_2O_2-induced increase in $[Na^+]_i$, at least in part.

Keywords: L-*cis*-Diltiazem; D-*cis*-Diltiazem; Hydrogen peroxide; Energy metabolism; Na^+, intracellular; NMR (Nuclear magnetic resonance)

1. Introduction

Diltiazem (D-*cis*-diltiazem), a benzothiazepine Ca^{2+} channel blocker, has been used for the treatment of ischemic heart disease. The primary mechanism of the anti-ischemic action of D-*cis*-diltiazem is considered to be its Ca^{2+} channel blocking action, which produces improvement of the myocardial oxygen balance between supply and demand by either increasing coronary flow or decreasing cardiac mechanical function, or both. However, L-*cis*-diltiazem, which is an optical isomer of D-*cis*-diltiazem with about 1/20–1/100 the Ca^{2+} channel blocking action of D-*cis*-diltiazem (Nasa et al., 1992; Itogawa et al., 1996), and D-*cis*-diltiazem attenuate the myocardial derangements induced by ischemia–reperfusion to a similar degree (Nasa et al., 1990). This fact suggests that the anti-ischemic action of D-*cis*-diltiazem does not entirely depend on its Ca^{2+} channel blocking action, but depends on other unknown actions. Recently, we (Xiao et al., 1997) have demonstrated that both D-*cis*- and L-*cis*-diltiazem attenuate the myocardial derangements induced by palmitoyl-L-carnitine, which accumulates in the heart during ischemia and reperfusion, supporting the above suggestion.

During ischemia and reperfusion, reactive oxygen species, such as superoxide anion, hydroxyl radical and hydrogen peroxide (H_2O_2), are generated intra- and extracellularly in the myocardium and endothelium (Hess and Manson, 1984; Lucchesi, 1990; Loesser et al., 1991). These reactive oxygen species are considered important in producing irreversible damage in the ischemia-reperfused heart (Hess and Manson, 1984; Lucchesi, 1990; Loesser et al., 1991). In fact, oxygen radical scavengers and anti-oxidants protect the myocardium against ischemia–reperfusion damage (Hess and Manson, 1984; Lucchesi, 1990; Loesser et al., 1991). We (Hara et al., 1993; Hara and Abiko, 1996; Kokita and Hara, 1996) have demonstrated that H_2O_2 decreases cardiac mechanical function and the tissue levels of high-energy phosphates in the isolated perfused rat heart. According to recent physiological studies, H_2O_2 increases the intracellular concentration of Na^+ ($[Na^+]_i$) in the myocardium (Yanagida et al., 1995), probably because of an increase in Na^+ current in cardiac cells (Bhatnagar et al., 1990; Ward and Giles, 1997). The

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increase in $[Na^+]_i$ activates the Na^+/Ca^{2+} exchanger, leading to intracellular Ca^{2+} overload, which is responsible for irreversible myocardial derangements (Goldhaber, 1996). In order to elucidate the mechanisms of action of L-*cis*-diltiazem responsible for its anti-ischemic effect and to relate its anti-ischemic action to the Ca^{2+} channel blocking action, the following two series of experiments were designed. The first series of experiments was performed to determine the effect of D-*cis*- and L-*cis*-diltiazem on the H_2O_2-induced mechanical dysfunction, decrease in the tissue levels of high-energy phosphates and damage of the cell membrane, and the second series of experiments was carried out to examine the effect of D-*cis*- and L-*cis*-diltiazem on the H_2O_2-induced increase in $[Na^+]_i$.

2. Methods

The protocol of animal experiments in the present study was approved by the 'Asahikawa Medical College Committee on Animal Research'.

2.1. Heart perfusion (the first series of experiments)

Male Sprague–Dawley rats, weighing 310–340 g (9–10 weeks old), were used in the first series of experiments. The rats were anesthetized with sodium pentobarbital (50 mg kg^{-1}, i.p.) 20 min after an injection with heparin (1000 u kg^{-1}, i.p.). After thoracotomy, the hearts were rapidly removed, and then retrograde perfusion was started using a cannula inserted into the aorta according to the Langendorff technique. The perfusion buffer was a Krebs–Henseleit bicarbonate (KHB) buffer containing (in mM): NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25; and glucose, 11, equilibrated with a gas mixture of 95% O_2 + 5% CO_2 and maintained at 37°C. The oxygen tension of the buffer measured by a blood gas analyzer (Model 813, Instrumentation Laboratory, Lexington, USA) was about 550 mm Hg. The hearts, mounted in a water-jacketed chamber (37°C), were initially perfused at a constant perfusion pressure of 80 cm H_2O. About 10 min after constant pressure perfusion, perfusion was switched to constant flow perfusion (10 ml min^{-1}), using a microtube pump (Eyela MP-3, Tokyo-Rikakikai Instruments, Tokyo, Japan), which was maintained until the end of the experiment. The heart rate was kept constant by pacing the heart at 300 beats min^{-1} with an electronic stimulator (3F46, San-Ei Instruments, Tokyo, Japan) during the course of the study. Rectangular pulses of 2 ms in duration with a voltage of 6 V (about 3 × the threshold voltage) were applied to the left ventricle for pacing of the heart.

As indices of mechanical function, left ventricular systolic pressure, left ventricular end-diastolic pressure and left ventricular developed pressure were used. The values of left ventricular systolic pressure and left ventricular end-diastolic pressure were determined from the left ventricular pressure curves recorded continuously during the course of the study, and the left ventricular developed pressure value was calculated as left ventricular systolic pressure minus left ventricular end-diastolic pressure. For measurement of left ventricular pressure, a saline-filled polyethylene cannula, connected to a pressure transducer, was inserted into the left ventricular cavity via the left atrium.

2.2. Experimental protocol (the first series of experiments)

The hearts were divided into five groups: vehicle, L-*cis*-diltiazem (5 μM), L-*cis*-diltiazem (10 μM), L-*cis*-diltiazem (15 μM) and D-*cis*-diltiazem (15 μM) groups. In these groups, the hearts were perfused at a constant flow for 75 min (stabilization period for 20 min and observation period for 55 min). D-*cis*-Diltiazem, L-*cis*-diltiazem or vehicle (KHB buffer for both D-*cis*-diltiazem and L-*cis*-diltiazem solutions) was infused into the aortic cannula at a constant flow rate of 0.1 ml min^{-1} for 45 min from 10 min after the start of observation period. The final concentration of D-*cis*-diltiazem in the perfusate was set to 15 μM and that of L-*cis*-diltiazem in the perfusate was set to 5, 10 or 15 μM. H_2O_2 was infused into the aortic cannula at a constant flow rate of 0.1 ml min^{-1} for 3.5 min from 10 min after the start of infusion of D-*cis*-diltiazem, L-*cis*-diltiazem or vehicle. The final concentration of H_2O_2 in the perfusate was set to 600 μM. During the observation period, left ventricular systolic pressure and left ventricular end-diastolic pressure were continuously recorded and the coronary effluent was collected for determination of lactate dehydrogenase (LDH) released from the myocardium. To measure the tissue levels of high-energy phosphates, the hearts were frozen at the end of experiment (45 min after the start of infusion of vehicle, D-*cis*-diltiazem or L-*cis*-diltiazem) with freezing clamps previously chilled in liquid nitrogen. In addition, some hearts in the vehicle, D-*cis*-diltiazem (15 μM) and L-*cis*-diltiazem (15 μM) groups were frozen immediately before H_2O_2 infusion (10 min after the start of infusion of vehicle, D-*cis*-diltiazem or L-*cis*-diltiazem). The frozen myocardial samples were stored in liquid nitrogen (at −196°C) until the biochemical analysis was performed.

2.3. Biochemical analysis (the first series of experiments)

The frozen myocardial sample was pulverized in a mortar cooled with liquid nitrogen. A part of the pulverized tissue powder (about 0.8–1.0 g) was weighed and put into an oven overnight, in order to measure the tissue water content and dry weight of the tissue. The remainder of the tissue powder was used for determination of the tissue levels of ATP, ADP, AMP and creatine phosphate. ATP, ADP, AMP and creatine phosphate were measured according to standard enzymatic procedures (Bergmeyer, 1974a), using a spectrophotometer (Gilford system 2600,

Gilford Instrument Laboratories, Oberlin, USA). The LDH activity in the coronary effluent was measured spectrophotometrically according to an enzymatic method (Bergmeyer, 1974b), using an LDH assay kit (Sigma, St. Louis, USA).

2.4. Heart perfusion (the second series of experiments)

Male Sprague–Dawley rats, weighing 180–210 g (6–7 weeks old), were used in the second series of experiments. According to the procedure described in the first series of experiments, the isolated hearts were initially perfused by the Langendorff technique at a constant perfusion pressure of 80 cm H_2O. About 10 min after constant pressure perfusion, perfusion was switched to constant flow perfusion (8.5 ml min^{-1}), using a microtube pump (Eyela MP-3, Tokyo-Rikakikai Instruments), which was maintained until the end of the experiment. During the first 10 min of constant flow perfusion, the hearts were perfused with normal KHB buffer. To obtain ^{23}Na-nuclear magnetic resonance (NMR) spectra, perfusion buffer was then switched to a KHB buffer containing thulium (III)-1, 4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis-(methylenephosphonate) [$Tm(DOTP)^{5-}$] (a ^{23}Na-NMR shift reagent), equilibrated with a gas mixture of 95% O_2 + 5% CO_2 and maintained at 37°C. The buffer for the study with ^{23}Na-NMR spectra was the same as the normal KHB buffer, except that it contained 104 mM NaCl, 3.5 mM $Na_4HTmDOTP$ and 3.9 mM $CaCl_2$. The reason for use of 3.9 mM $CaCl_2$ was that addition of 3.9 mM $CaCl_2$ would produce 1 mM free Ca^{2+} in the presence of the shift reagent (Buster et al., 1990). Although the concentration of free Ca^{2+} was lower than that used in the first series of experiments (2.5 mM), it (1 mM) was chosen to avoid precipitation of the shift reagent (Van Emous et al., 1997). The heart rate was kept constant by pacing the heart at 300 beats min^{-1}, using a pair of copper electrodes and an electronic stimulator (SEN-3301, Nihon Kohden, Tokyo, Japan). For measurement of left ventricular pressure, a latex balloon connected to a pressure transducer was inserted into the left ventricular cavity via the left atrium. Before experiments, the balloon was inflated with distilled water to achieve an initial end-diastolic pressure of 10 mm Hg; thereafter, the volume was kept constant during the course of the study. The hearts perfused with the buffer were placed inside a glass NMR tube (13.4 mm in internal diameter) that was introduced into the NMR probe placed in a superconducting magnet. Because the internal diameter of the glass NMR tube was small, only small hearts were used for the NMR experiments. Therefore, relatively small rats weighing about 200 g were used in the second series of experiments. The temperature surrounding the glass NMR tube was maintained at about 20°C by air conditioner, because the apparatus used in the present study is not equipped to provide a temperature-controlled air stream around the NMR tube. Coronary effluent was removed from the NMR glass tube to a level of about 10 mm above the heart. Perfusion fluid was not recirculated.

2.5. ^{23}Na-NMR measurements (the second series of experiments)

^{23}Na-spectra were obtained using a GX-270 spectrometer (JEOL, Tokyo, Japan) at 71.32 MHz with a pulse width of 90°, an interpulse delay of 0.5 s, a spectral width of 3000 Hz and data size of 2048. The spectrometer was equipped with a 6.3-T superconducting magnet and an NMR probe (TU (15), JEOL) in which the glass NMR tube was placed. Four glass capillaries, each containing 1.67 μmol NaCl in the presence of 50 mM Tris form of dysprosium triethylenetetraminehexaacetic acid [$Tris_3$-Dy(TTHA) · 3Tris–HCl] (another ^{23}Na-NMR shift reagent), were placed inside of the NMR tube as an internal standard. The area of each peak in the ^{23}Na-NMR spectra was analyzed by integration after Gaussian and Lorentzian multiplication and baseline correction using JEOL ALICE2 software (JEOL DATUM, Tokyo, Japan). The $[Na^+]_i$ of the cardiac cells was calculated by a comparison between the peak area of $[Na^+]_i$ and that of the reference solution in the glass capillaries.

2.6. Experimental protocol (the second series of experiments)

The hearts were divided into four groups: vehicle, D-*cis*-diltiazem (15 μM), L-*cis*-diltiazem (15 μM) and tetrodotoxin (3 μM) groups. In these groups, the hearts were perfused with the $Tm(DOTP)^{5-}$-containing KHB buffer throughout the experiment (stabilization period for 10 min and observation period for 40 min). After the stabilization period, D-*cis*-diltiazem, L-*cis*-diltiazem or vehicle (KHB buffer for both D-*cis*-diltiazem and L-*cis*-diltiazem) was infused into the aortic cannula for 40 min at a constant flow rate of 0.1 ml min^{-1}. The final concentration of D-*cis*-diltiazem and L-*cis*-diltiazem in the perfusate was set to 15 μM and the final concentration of tetrodotoxin in the perfusate was set to 3 μM. H_2O_2 was infused into the aortic cannula at a constant flow rate of 0.1 ml min^{-1} for 4 min from 10 min after the start of infusion of L-*cis*-diltiazem, D-*cis*-diltiazem or tetrodotoxin. The final concentration of H_2O_2 in the perfusate was set to 1 mM. Thus, the concentration of H_2O_2 used in the second series of experiments was higher than that used in the first series of experiments, because in a preliminary experiment, a high concentration of H_2O_2 was needed to induce mechanical dysfunction to a degree similar to that in the first series of experiments. After the experiment, the hearts were put into an oven (80°C) for 48 h, in order to measure the dry weight of the tissue. The volume of intracellular water was assumed to be 2.45 ml g^{-1} dry weight (Askenasy et al., 1995).

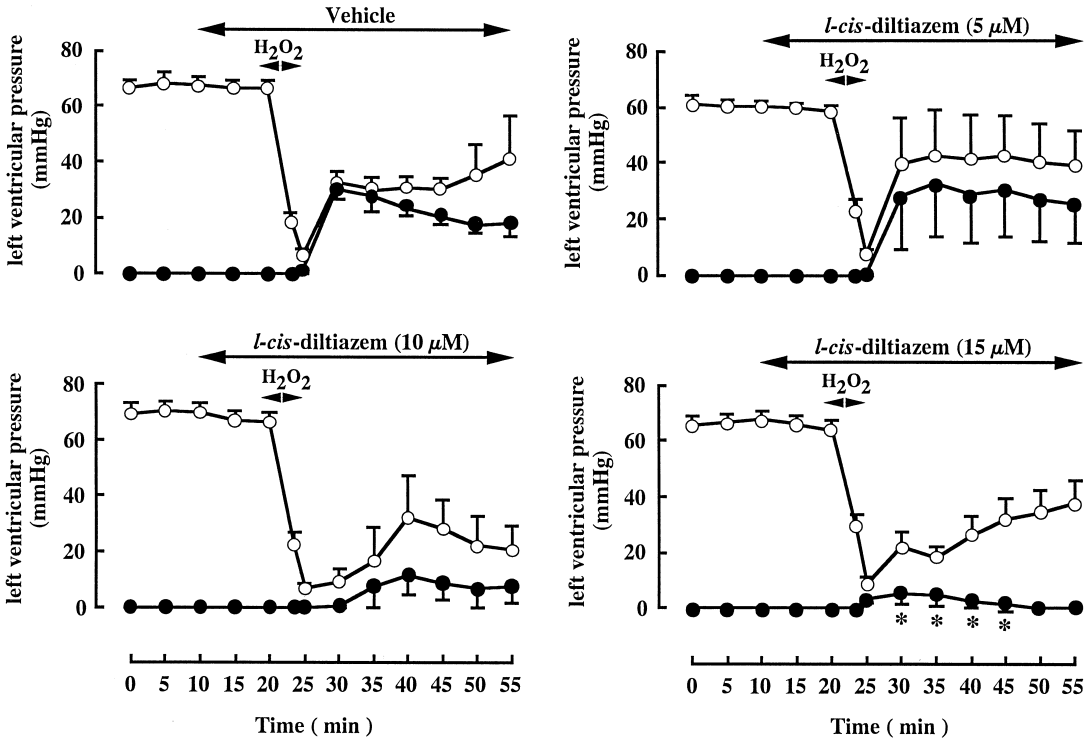

Fig. 1. Effects of various concentrations of L-*cis*-diltiazem (5, 10 and 15 μM) on the H_2O_2-induced changes in mechanical function. The open circle indicates left ventricular systolic pressure and the solid circle indicates left ventricular end-diastolic pressure. Each value represents the mean ± S.E.M. ($n = 4–6$). $^*P < 0.05$ when compared with the value in the vehicle group.

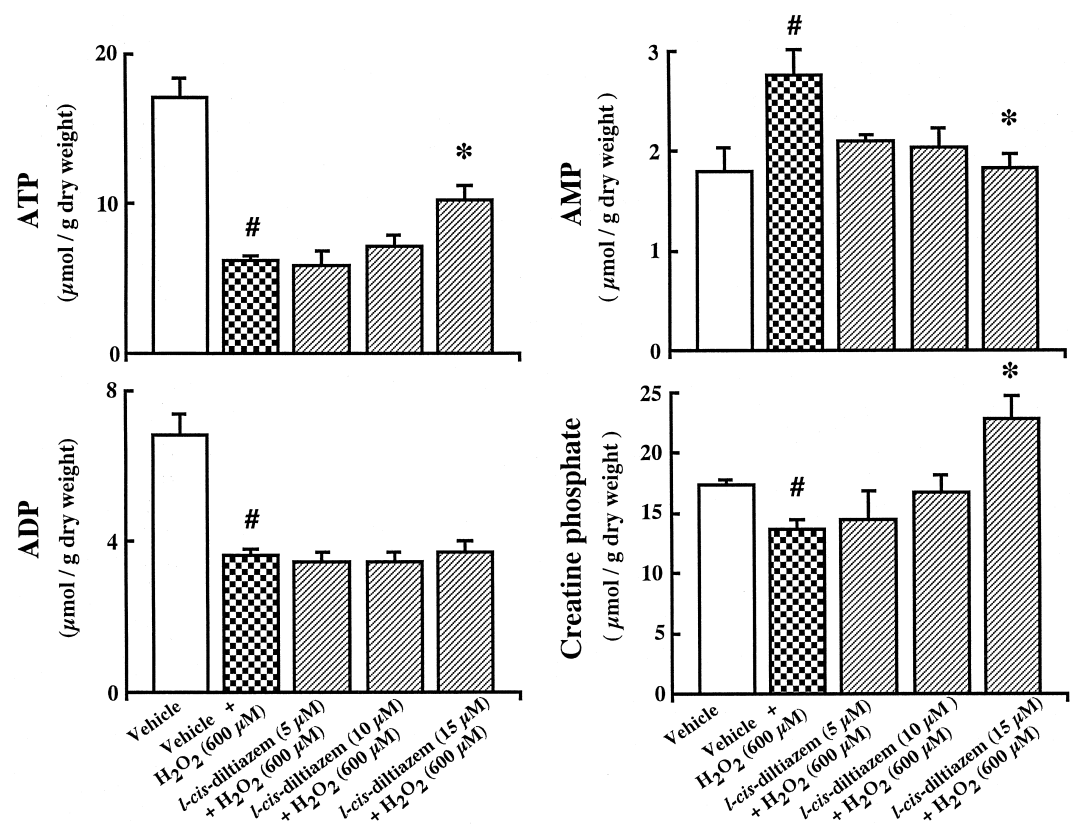

Fig. 2. Effects of various concentrations of L-*cis*-diltiazem (5, 10 and 15 μM) on the H_2O_2-induced changes of the tissue levels of ATP, ADP, AMP and creatine phosphate, which were measured at the end of the experiment (45 min after the start of vehicle or L-*cis*-diltiazem infusion). Each value represents the mean ± S.E.M. ($n = 4–6$). $^{\#}P < 0.05$ when compared with the value in the vehicle group in the H_2O_2-untreated heart; $^*P < 0.05$ when compared with the value in the vehicle group in the H_2O_2-treated heart.

Table 1
Effects of D-*cis*- or L-*cis*-diltiazem on the concentration of H_2O_2 in the buffer solution in vitro. For each concentration of H_2O_2 (measured), data represent the means ± S.E.M. of four samples for each experiment. The mixture of H_2O_2 (600 μM) and D-*cis*-diltiazem (15 μM), L-*cis*-diltiazem (15 μM) or dimethylthiourea (10 mM) in the 0.1 M phosphate buffer (pH 7.0) was incubated at 37°C for 30 min. The H_2O_2 concentration was estimated from the absorbance

Mixture	H_2O_2 concentration (measured; μM)
H_2O_2 (600 μM) + vehicle	606.4 ± 3.5
H_2O_2 (600 μM) + D-*cis*-diltiazem (15 μM)	608.1 ± 4.1
H_2O_2 (600 μM) + L-*cis*-diltiazem (15 μM)	610.6 ± 3.9
H_2O_2 (600 μM) + dimethylthiourea (10 mM)	274.1 ± 4.8[a]

[a] $P < 0.05$ when compared with the value in the vehicle group.

2.7. Direct effect of D-cis-diltiazem and L-cis-diltiazem on the H_2O_2 concentration in vitro (another experiment)

To determine whether D-*cis*- and L-*cis*-diltiazem have a direct scavenging effect on H_2O_2, the effect of D-*cis*-diltiazem and L-*cis*-diltiazem on the H_2O_2 concentration was investigated and compared with that of dimethylthiourea, which has an H_2O_2-scavenging action (Jackson et al., 1985). A mixture of H_2O_2 (600 μM) and D-*cis*- or L-*cis*-diltiazem (15 μM) or that of H_2O_2 (600 μM) and dimethylthiourea (10 mM) in 0.1 M phosphate buffer (pH 7.0) was incubated at 37°C for 30 min. The H_2O_2 concentration in the buffer was measured according to a spectrophotometric method (Hayashi et al., 1989).

2.8. Drugs

D-*cis*-Diltiazem (Sigma), L-*cis*-diltiazem (Tanabe Seiyaku, Osaka, Japan) and tetrodotoxin (Sankyo, Tokyo, Japan) were dissolved in KHB buffer solution. These agents were infused at a flow rate of 0.1 ml min^{-1}, using an infusion pump, into the inflow tube connected to the side arm of the aortic cannula. H_2O_2 (Nacalai Tesque, Kyoto, Japan) was diluted with saline solution. H_2O_2 was also infused into the inflow tube at a flow rate of 0.1 ml min^{-1} using another infusion pump. Dimethylthiourea was purchased from Aldrich Chemical (St. Louis, USA). The reagents and enzymes used for biochemical analysis were purchased from Sigma. $Na_4HTmDOTP$ was purchased from Magnetic Resonance Solutions (Dallas, USA). $Tris_3Dy(TTHA) \cdot 3Tris–HCl$ (50 mM) was prepared by mixing $DyCl_3 \cdot 6H_2O$ (Sigma) (50 mM) with H_6TTHA (Sigma) (50 mM) in the Tris buffer (pH 7.4).

2.9. Statistical analysis

All values are expressed as means ± S.E.M. When changes in left ventricular systolic pressure, left ventricular end-diastolic pressure, left ventricular developed pressure, LDH release and $[Na^+]_i$ were compared between vehicle-treated and drug-treated groups, statistical analysis was performed with a two-way repeated measures analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. If a significant difference was obtained between these groups, further comparisons at each time point were performed by means of Dunnett's test (Figs. 1, 3, 4, 6 and 8). When the tissue levels of energy metabolites in the vehicle group were compared between H_2O_2-untreated and H_2O_2-treated hearts, unpaired Student's *t*-test was used (Figs. 2 and 5). When the tissue levels of energy metabolites and the H_2O_2 concentration were compared between vehicle-treated and drug-treated groups, statistical analysis was performed with a one-way ANOVA followed by Dunnett's test for multiple comparisons (Figs. 2 and 5, Table 1). A difference was considered statistically significant at $P < 0.05$.

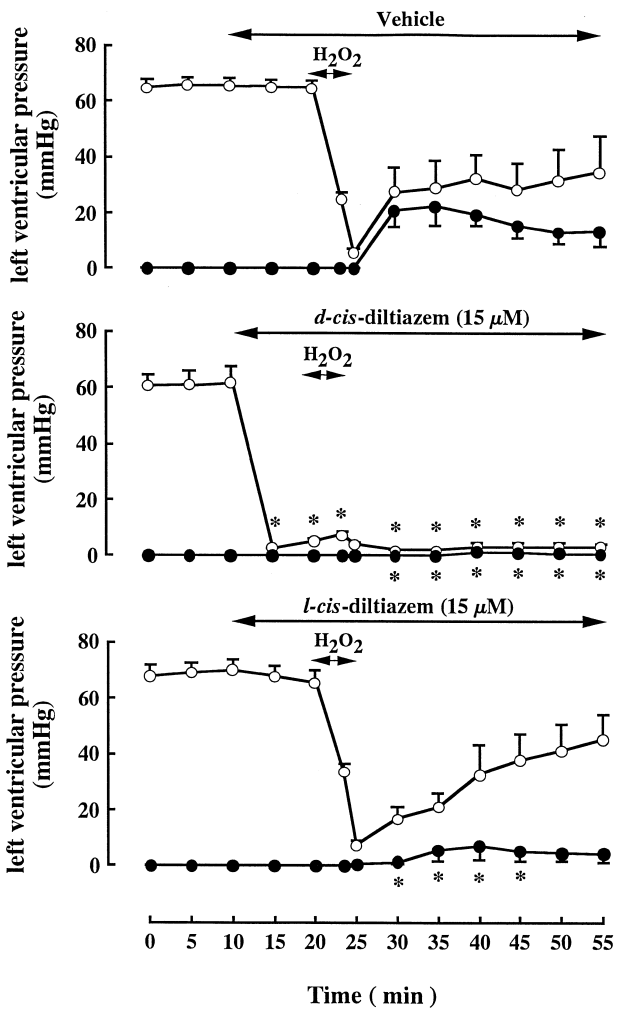

Fig. 3. Effects of D-*cis*-diltiazem (15 μM) or L-*cis*-diltiazem (15 μM) on the H_2O_2-induced changes in mechanical function. The open circle indicates left ventricular systolic pressure and the solid circle indicates left ventricular end-diastolic pressure. Each value represents the mean ± S.E.M. ($n = 7$). *$P < 0.05$ when compared with the value in the vehicle group.

3. Results

3.1. Effects of D-cis- and L-cis-diltiazem on H_2O_2-induced mechanical derangements in the heart

Fig. 1 shows the effect of various concentrations of L-*cis*-diltiazem (5, 10 or 15 μM) on the H_2O_2-induced mechanical dysfunction. Before the start of the infusion of L-*cis*-diltiazem (0, 5 and 10 min in Fig. 1), there was no significant difference in the values of left ventricular systolic pressure and left ventricular end-diastolic pressure between the vehicle and L-*cis*-diltiazem groups. No concentration of L-*cis*-diltiazem (5, 10 or 15 μM) had a significant action on left ventricular systolic pressure and left ventricular end-diastolic pressure before H_2O_2 infusion (20 min in Fig. 1). In the vehicle group, H_2O_2 produced a temporary, but marked decrease in left ventricular systolic pressure ($P < 0.001$ by ANOVA), which was followed by incomplete recovery to the initial level. H_2O_2 also produced a marked increase in left ventricular end-diastolic pressure ($P < 0.001$ by ANOVA), which was accompanied by an increase in left ventricular systolic pressure. The decrease in left ventricular systolic pressure induced by H_2O_2 was not significantly modified by any concentration of L-*cis*-diltiazem ($P = 0.616$ by ANOVA). In contrast, a high concentration of L-*cis*-diltiazem (15 μM) significantly attenuated the H_2O_2-induced increase in left ventricular end-diastolic pressure ($P < 0.05$ by ANOVA followed by Dunnett's test), although lower concentrations of L-*cis*-diltiazem (5 or 10 μM) did not.

Fig. 2 shows the effects of various concentrations of L-*cis*-diltiazem (5, 10 or 15 μM) on the H_2O_2-induced decrease in the tissue levels of high-energy phosphates. In the vehicle group, H_2O_2 decreased the tissue levels of ATP, ADP and creatine phosphate and increased the tissue level of AMP ($P < 0.05$). These changes in the tissue levels of high-energy phosphates were not attenuated by lower concentrations of L-*cis*-diltiazem (5 or 10 μM). However, a high concentration of L-*cis*-diltiazem (15 μM) significantly attenuated the H_2O_2-induced changes in the tissue levels of ATP, AMP and creatine phosphate ($P < 0.05$ by ANOVA followed by Dunnett's test). These results suggest that at the concentration of 15 μM, L-*cis*-diltiazem attenuates the H_2O_2-induced mechanical and metabolic derangements in the heart. Next, the effect of L-*cis*-diltiazem (15 μM) was compared with that of D-*cis*-diltiazem (15 μM).

Fig. 3 shows the effects of D-*cis*-diltiazem (15 μM) and L-*cis*-diltiazem (15 μM) on the H_2O_2-induced changes in left ventricular systolic pressure and left ventricular end-diastolic pressure. Before the infusion of D-*cis*- or L-*cis*-diltiazem (0, 5 and 10 min in Fig. 3), there was no significant difference in the values of left ventricular systolic pressure and left ventricular end-diastolic pressure among vehicle, D-*cis*-diltiazem and L-*cis*-diltiazem groups. Before the H_2O_2 infusion (15 and 20 min in Fig. 3), there was a significant decrease in the left ventricular systolic pressure in the D-*cis*-diltiazem group ($P < 0.05$ by ANOVA followed by Dunnett's test), while there was no decrease in the L-*cis*-diltiazem group; left ventricular systolic pressure decreased only in the D-*cis*-diltiazem group (15–55 min in Fig. 3). As seen in Fig. 1, in the vehicle group H_2O_2 decreased left ventricular systolic pressure ($P < 0.001$ by ANOVA) and increased left ventricular end-diastolic pres-

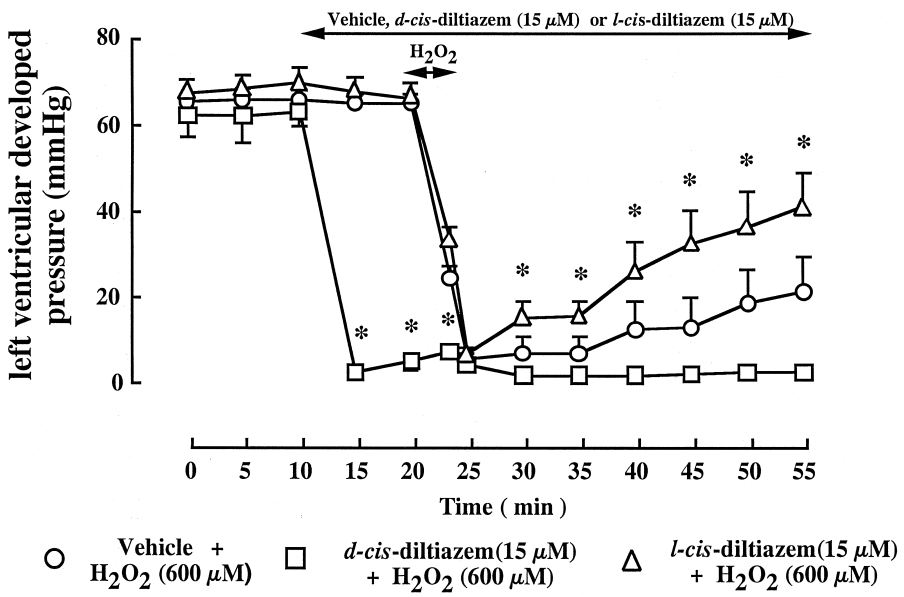

Fig. 4. Effects of D-*cis*-diltiazem (15 μM) or L-*cis*-diltiazem (15 μM) on the H_2O_2-induced changes in left ventricular developed pressure, which is expressed as left ventricular systolic pressure minus left ventricular end-diastolic pressure. Values were calculated from the values of left ventricular systolic pressure and left ventricular end-diastolic pressure in Fig. 3. Each value represents the mean ± S.E.M. ($n = 7$). *$P < 0.05$ when compared with the value in the vehicle group.

sure ($P < 0.001$ by ANOVA). It is noteworthy, however, that both D-*cis*- and L-*cis*-diltiazem attenuated significantly the H_2O_2-induced increase in left ventricular end-diastolic pressure ($P < 0.05$ by ANOVA followed by Dunnett's test).

Fig. 4 shows the effects of D-*cis*-diltiazem (15 μM) and L-*cis*-diltiazem (15 μM) on the H_2O_2-induced decrease in left ventricular developed pressure, which is expressed as left ventricular systolic pressure minus left ventricular end-diastolic pressure. Before the infusion of D-*cis*- or L-*cis*-diltiazem (0, 5 and 10 min in Fig. 4), there was no significant difference in the left ventricular developed pressure value among vehicle, D-*cis*- and L-*cis*-diltiazem groups. Before the H_2O_2 infusion (15 and 20 min in Fig. 4), there was a significant decrease in the left ventricular developed pressure in the D-*cis*-diltiazem group ($P < 0.05$ by ANOVA followed by Dunnett's test), while there was no decrease in the L-*cis*-diltiazem group; D-*cis*-diltiazem alone decreased left ventricular developed pressure (15–55 min in Fig. 4). In the vehicle group, H_2O_2 decreased left ventricular developed pressure markedly ($P < 0.001$ by ANOVA). L-*cis*-Diltiazem attenuated significantly the

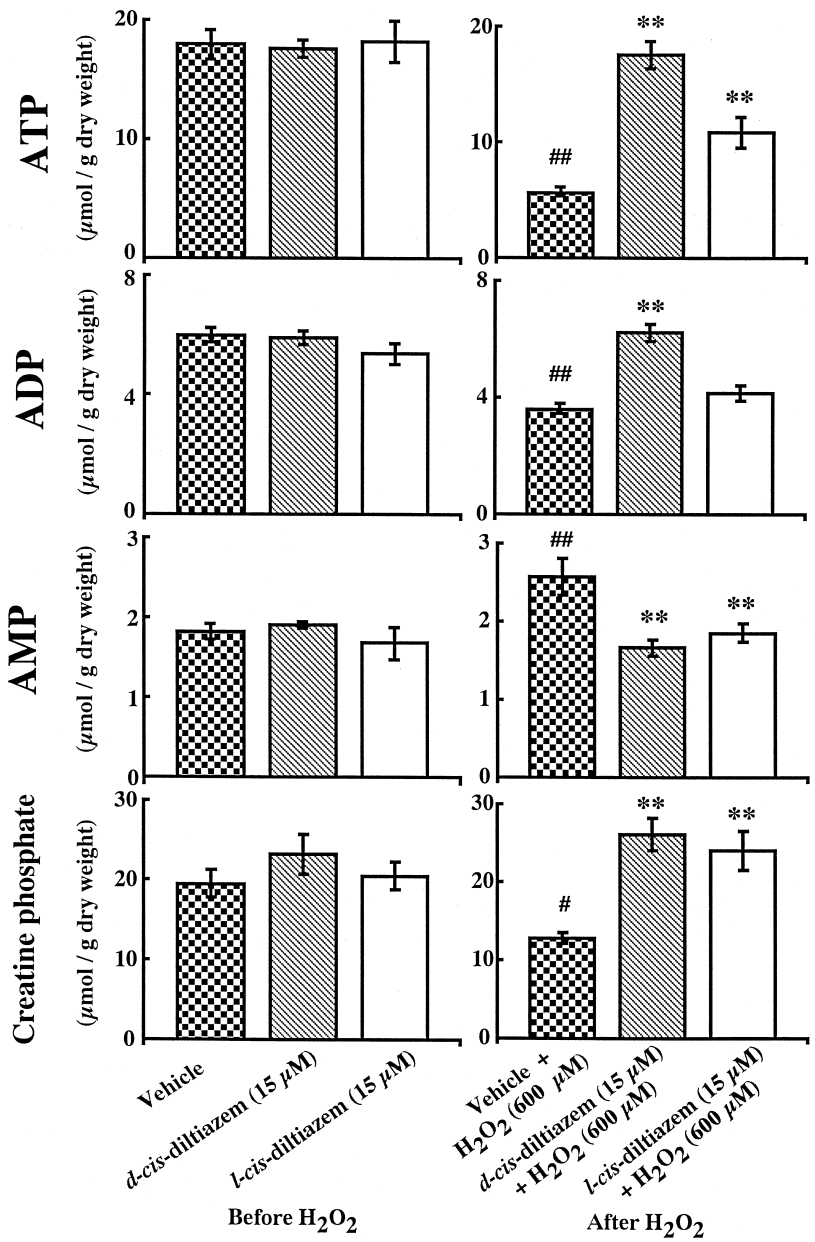

Fig. 5. Effects of D-*cis*-diltiazem (15 μM) or L-*cis*-diltiazem (15 μM) on the H_2O_2-induced changes in tissue levels of ATP, ADP, AMP and creatine phosphate, which were measured immediately before H_2O_2 infusion (10 min after the start of vehicle, D-*cis*-diltiazem or L-*cis*-diltiazem infusion) and at the end of the experiment (45 min after the start of vehicle, D-*cis*-diltiazem or L-*cis*-diltiazem infusion). Hearts used for values of 'after H_2O_2' are those in Fig. 3. Each value represents the mean ± S.E.M. ($n = 4–7$). $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ when compared with the value in the vehicle group in the H_2O_2-untreated heart; $^{**}P < 0.01$ when compared with the value in the vehicle group in the H_2O_2-treated heart.

H_2O_2-induced decrease in left ventricular developed pressure ($P < 0.001$ by ANOVA followed by Dunnett's test), whereas D-*cis*-diltiazem did not attenuate it, probably because of its cardiodepressive action. Thus, L-*cis*-diltiazem (15 μM) attenuated the H_2O_2-induced changes in both left ventricular developed pressure and left ventricular end-diastolic pressure.

Fig. 5 shows the tissue levels of ATP, ADP, AMP and creatine phosphate in the vehicle, D-*cis*-diltiazem (15 μM) and L-*cis*-diltiazem (15 μM) groups before H_2O_2 infusion (20 min in Fig. 4) and after the H_2O_2 infusion (55 min in Fig. 4). The tissue levels of ATP, ADP, AMP and creatine phosphate before H_2O_2 infusion were similar among the vehicle, D-*cis*-diltiazem and L-*cis*-diltiazem groups ($P > 0.05$ by ANOVA). In the vehicle group, H_2O_2 decreased the tissue levels of ATP, ADP and creatine phosphate, and increased the tissue level of AMP ($P < 0.05$). These changes in the tissue levels of energy metabolites induced by H_2O_2 were significantly attenuated by D-*cis*-diltiazem ($P < 0.01$ by ANOVA followed by Dunnett's test). L-*cis*-Diltiazem also attenuated the H_2O_2-induced changes in the tissue levels of ATP, AMP and creatine phosphate ($P < 0.01$ by ANOVA followed by Dunnett's test), although it failed to attenuate the H_2O_2-induced decrease in the tissue level of ADP. These results suggest that both D-*cis*- and L-*cis*-diltiazem attenuate the H_2O_2-induced decrease in high-energy phosphates in the heart.

Fig. 6 shows the effects of D-*cis*-diltiazem (15 μM) and L-*cis*-diltiazem (15 μM) on the H_2O_2-induced release of LDH. In the vehicle, D-*cis*-diltiazem and L-*cis*-diltiazem groups, there was no significant difference in the release of LDH before H_2O_2 infusion. In the vehicle group, H_2O_2 produced a marked release of LDH from the myocardium into the effluent ($P < 0.001$ by ANOVA); the LDH release reached a maximum 21.5 min after the end of H_2O_2 infusion (35 min in Fig. 6). The LDH release induced by H_2O_2 was significantly attenuated by D-*cis*- or L-*cis*-diltiazem ($P < 0.05$ by ANOVA followed by Dunnett's test). These results suggest that both D-*cis*- and L-*cis*-diltiazem attenuate the H_2O_2-induced damage of the cardiac cell membrane.

3.2. Effect of D-cis- and L-cis-diltiazem on H_2O_2-induced changes in $[Na^+]_i$

Fig. 7 shows typical ^{23}Na spectra for the rat heart in the presence of the ^{23}Na shift reagent $Tm(DOTP)^{5-}$. H_2O_2 (1 mM) was added to the heart for 4 min (from 15 to 19 min in Fig. 7). It is evident that in the vehicle group, H_2O_2 caused an increase in the peak of $[Na^+]_i$, which was markedly attenuated by D-*cis*-diltiazem (15 μM), L-*cis*-diltiazem (15 μM) or tetrodotoxin (3 μM). Fig. 8 shows the effects of D-*cis*-diltiazem (15 μM), L-*cis*-diltiazem (15 μM) and tetrodotoxin (3 μM) on the H_2O_2-induced increase in $[Na^+]_i$ in myocardial cells. In the vehicle group, H_2O_2 (1 mM) increased $[Na^+]_i$ markedly ($P < 0.001$ by ANOVA) and the increase reached a maximum 20 min after the end of H_2O_2 infusion (34 min in Fig. 8). The increase in $[Na^+]_i$ induced by H_2O_2 was almost completely inhibited by D-*cis*- or L-*cis*-diltiazem ($P < 0.05$ by ANOVA followed by Dunnett's test). Tetrodotoxin also attenuated the H_2O_2-induced increase in $[Na^+]_i$ ($P < 0.05$ by ANOVA followed by Dunnett's test). These results suggest that both D-*cis*- and L-*cis*-diltiazem attenuate the intracellular accumulation of intracellular Na^+ induced by H_2O_2. In the second series of experiments, we also continuously recorded the mechanical function (left ventricular pressure) of the heart, and similar results as in the first series of experiments (Fig. 3) were obtained: H_2O_2 decreased left ventricular systolic pressure and increased left ventricular end-diastolic pressure, and the H_2O_2-induced increase in left ventricular end-diastolic pressure was atten-

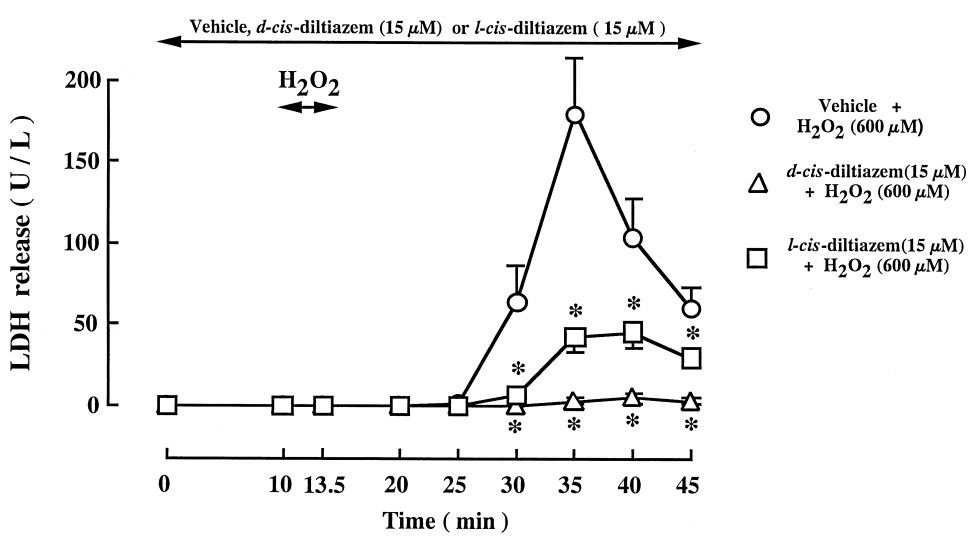

Fig. 6. Effects of D-*cis*-diltiazem (15 μM) or L-*cis*-diltiazem (15 μM) on the H_2O_2-induced changes in LDH release into effluent. Each value represents the mean ± S.E.M. ($n = 5$). *$P < 0.05$ when compared with the value in the H_2O_2 group.

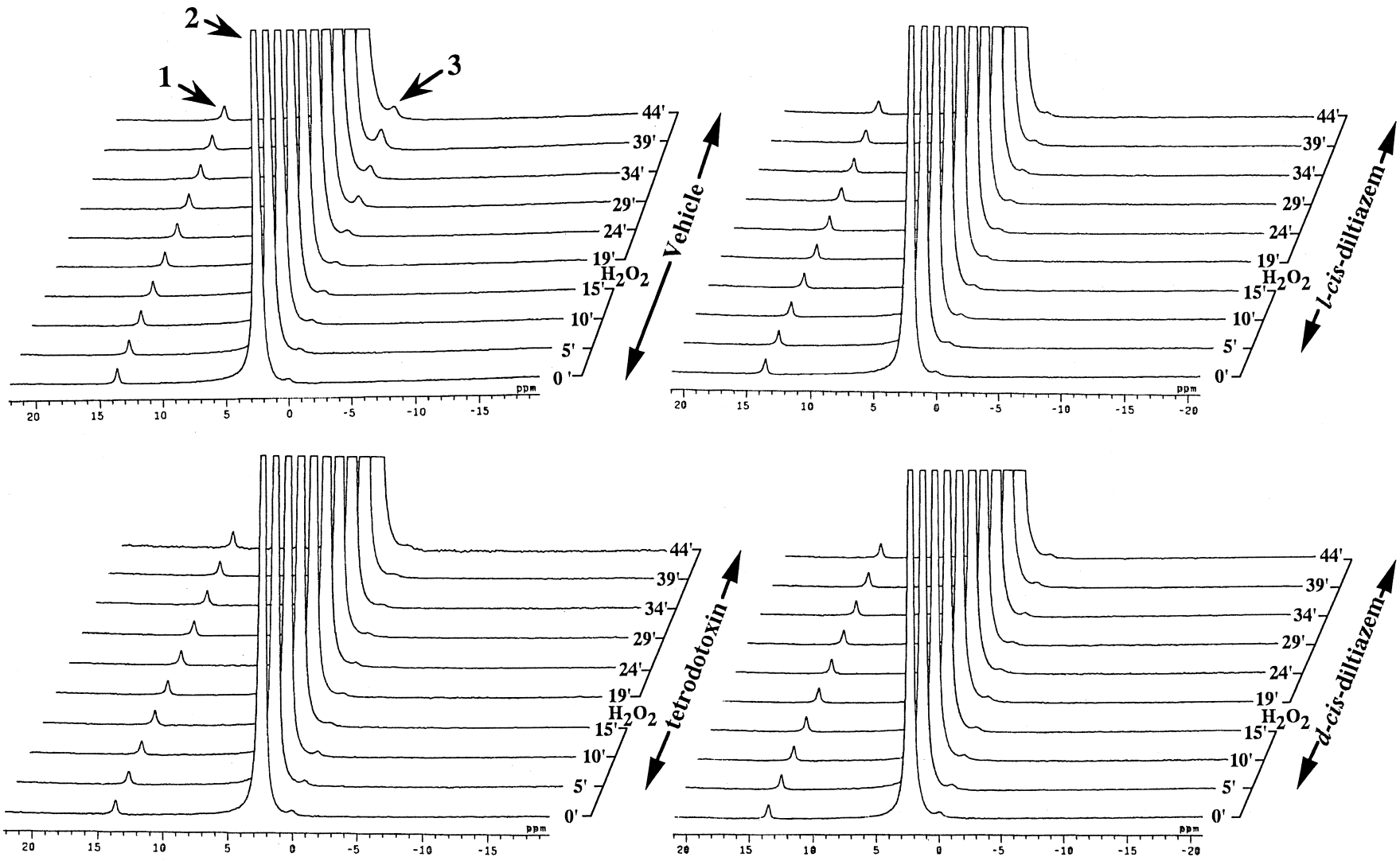

Fig. 7. Effects of D-*cis*-diltiazem (15 μM), L-*cis*-diltiazem (15 μM) and tetrodotoxin (3 μM) on the consecutive ^{23}Na-NMR spectra in the H_2O_2-treated heart. Typical ^{23}Na-NMR spectra in the vehicle, L-*cis*-diltiazem, tetrodotoxin and D-*cis*-diltiazem groups are shown. (1) The peak of the Na^+ reference (6.7 μmol Na^+ in total of the four capillary glasses) shifted with $Tris_3Dy(TTHA)\cdot 3Tris–HCl$; (2) the peak of the extracellular Na^+ (including bath Na^+) shifted with $Tm(DOTP)^{5-}$; (3) the peak of the unshifted intracellular Na^+.

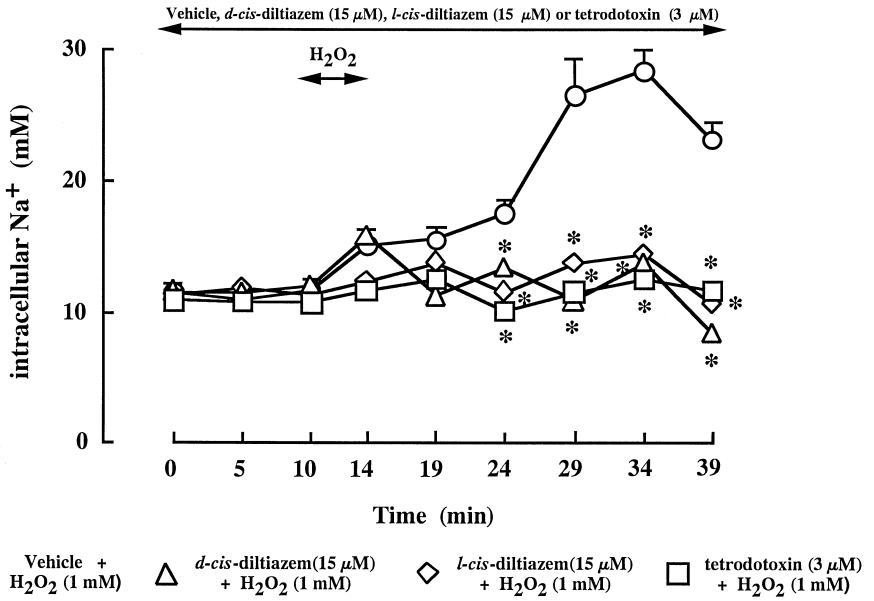

Fig. 8. Effects of D-*cis*-diltiazem (15 μM), L-*cis*-diltiazem (15 μM) or tetrodotoxin (3 μM) on the H_2O_2-induced changes in $[Na^+]_i$. $[Na^+]_i$ was calculated from ^{23}Na-NMR spectra in Fig. 7. Each value represents the mean ± S.E.M. ($n = 4–8$). *$P < 0.05$ when compared with the value in the vehicle group.

uated by D-*cis*-diltiazem (15 μM), L-*cis*-diltiazem (15 μM) or tetrodotoxin (data not shown).

If D-*cis*- and L-*cis*-diltiazem have a Na^+ channel blocking action, $[Na^+]_i$ in normal cardiac cells (without H_2O_2) should decrease after treatment of the heart with D-*cis*- or L-*cis*-diltiazem. However, neither tetrodotoxin, nor D-*cis*- and L-*cis*-diltiazem changed $[Na^+]_i$ in the normal cardiac cells: $[Na^+]_i$ remained unchanged within the accuracy of the assay.

3.3. Effect of D-cis- and L-cis-diltiazem on the concentration of H_2O_2 in the buffer solution in vitro

We examined whether D-*cis*- and L-*cis*-diltiazem have a direct scavenging effect on H_2O_2 in vitro. D-*cis*- or L-*cis*-Diltiazem, however, did not modify the H_2O_2 concentration in the buffer solution (Table 1). In contrast, dimethylthiourea, which has a H_2O_2-scavenging action, decreased the H_2O_2 concentration markedly. These results suggest that neither D-*cis*-diltiazem nor L-*cis*-diltiazem has a direct scavenging action on H_2O_2 in vitro.

4. Discussion

In the present study, we examined the effect of D-*cis*- and L-*cis*-diltiazem on the H_2O_2-induced myocardial derangements in the isolated perfused rat heart. L-*cis*-Diltiazem is an optical isomer of D-*cis*-diltiazem and is about 20–100 times less potent than D-*cis*-diltiazem in its Ca^{2+} channel blocking action (Nasa et al., 1992; Itogawa et al., 1996). We used H_2O_2 as a substance that may be responsible for ischemic derangements for the following reasons: (1) H_2O_2 and its metabolite, hydroxyl radical, are considered important in the pathogenesis of myocardial damage induced by ischemia–reperfusion (Brown et al., 1988; Loesser et al., 1991); (2) H_2O_2 penetrates the cell membrane and reaches the intracellular site (Fisher, 1988), and therefore it may produce severe damage to the cell.

In the first series of experiments, H_2O_2 produced an increase in left ventricular end-diastolic pressure and a decrease in left ventricular developed pressure (i.e., mechanical dysfunction), a decrease in the tissue levels of ATP and creatine phosphate and an increase in the tissue AMP level (i.e., energy deficiency), and an increase in LDH release from the myocardium (i.e., membrane damage). These alterations induced by H_2O_2 were attenuated by L-*cis*-diltiazem (15 μM). In contrast to L-*cis*-diltiazem, D-*cis*-diltiazem (15 μM) failed to attenuate the decrease in left ventricular developed pressure induced by H_2O_2, because D-*cis*-diltiazem has a potent Ca^{2+} channel blocking action, leading to a decrease in mechanical function. In fact, the cardiodepressive action of D-*cis*-diltiazem was observed before H_2O_2 infusion. These results suggest that both D-*cis*- and L-*cis*-diltiazem protect the myocardium against the H_2O_2-induced derangements, and that their cardioprotective action is not only due to the Ca^{2+} channel blocking action.

Some radical scavengers or anti-oxidants have been demonstrated to protect the myocardium against oxidative stress (Nakaya et al., 1987; Weglicki et al., 1990; Nagy et al., 1996). In fact, we have reported that, in the isolated perfused rat heart, catalase (a H_2O_2 scavenger) (Hara et al., 1993) or propofol (an intravenous anesthetic drug with an anti-oxidant action) (Kokita and Hara, 1996) attenuates the H_2O_2-induced mechanical dysfunction and the decrease in the tissue levels of high-energy phosphates. There is evidence to show that D-*cis*-diltiazem has an

anti-oxidant action (Janero et al., 1988; Mak and Weglicki, 1990; Weglicki et al., 1990), whereas there is no information about the anti-oxidant action of L-*cis*-diltiazem. The concentration of D-*cis*-diltiazem required for its anti-oxidant action is much higher than that used in the present study; in the sarcolemmal membrane, the concentration required to inhibit 50% of lipid peroxidation (IC_{50}) induced by oxygen radicals is 510 μM (Janero et al., 1988) or 850 μM (Mak and Weglicki, 1990). In the present study, neither D-*cis*-diltiazem (15 μM) nor L-*cis*-diltiazem (15 μM) modified the H_2O_2 concentration in the buffer solution in vitro. It is unlikely, therefore, that the protective effect of D-*cis*- and L-*cis*-diltiazem on the H_2O_2-induced derangements is due to the radical scavenging or anti-oxidant effect.

According to recent electrophysiological studies, H_2O_2 causes an increase in Na^+ current in ventricular myocytes, an increase which is blocked by tetrodotoxin (Bhatnagar et al., 1990; Ward and Giles, 1997). Ver Donck and Borgers (1991) have suggested that the reactive oxygen species causes excessive Na^+ entry through the fast Na^+ channel, leading to intracellular Ca^{2+} overload through the Na^+–Ca^{2+} exchange system, and hence myocardial damage. In fact, H_2O_2 increases intracellular concentrations of both Na^+ and Ca^{2+} in the myocardium (Yanagida et al., 1995). Interestingly, both D-*cis*- and L-*cis*-diltiazem have been demonstrated to block the Na^+ channel (Nakajima et al., 1975; Itogawa et al., 1996). Itogawa et al. (1996) reported that both D-*cis*- and L-*cis*-diltiazem inhibited the veratridine (a Na^+ channel opener)-induced increase in $[Na^+]_i$ and hypercontracture in rat myocytes at the concentration of 10 μM or higher. There is a possibility, therefore, that the beneficial effect of D-*cis*- and L-*cis*-diltiazem on the H_2O_2-induced derangements is due to their blocking effect on the Na^+ channel. To determine this possibility, we examined the effect of D-*cis*- and L-*cis*-diltiazem on the H_2O_2-induced increase in $[Na^+]_i$ in the myocardium using an NMR technique (the second series of experiments). We used a higher concentration of H_2O_2 in the second series of experiments than in the first series of experiments: in the second series of experiments, 1 mM H_2O_2 was applied for 4 min, whereas in the first series of experiments, 600 μM H_2O_2 was applied for 3.5 min. This is because in the second series of experiments, a large amount of H_2O_2 was needed to induce mechanical dysfunction to a degree similar to that seen in the first series of experiments. The decreased response of the heart to H_2O_2 in the second series of experiments may be due to the difference in perfusion conditions, such as lower Ca^{2+} concentration and lower temperature, which are determinants of cardioprotection; the concentration of free Ca^{2+} (1 mM) in the $Tm(DOTP)^{5-}$-containing KHB buffer used in the second series of experiments was lower than that (2.5 mM) in the KHB buffer (normal KHB buffer) used in the first series of experiments, and the temperature around the NMR tube (about 20°C) used in the second series of experiments was lower that in the water-jacketed chamber (37°C) used in the first series of experiments. When the membrane is severely damaged, intracellular Na^+ resonance collapses and a broad Na^+ resonance appears in the ^{23}Na-NMR spectra because of entry of the shift reagent into the cell, and therefore measurement of $[Na^+]_i$ is impossible (Jansen et al., 1998). As shown in Fig. 7, however, no broad Na^+ resonance was observed, suggesting that the measurement of $[Na^+]_i$ was appropriate in the present study.

The results of the second series of experiments indicate that both D-*cis*- and L-*cis*-diltiazem attenuate the H_2O_2-induced increase in $[Na^+]_i$ in the heart. Tetrodotoxin (3 μM), a specific inhibitor of Na^+ channels (Duff et al., 1988), also attenuated the H_2O_2-induced increase in $[Na^+]_i$ in the heart and the H_2O_2-induced myocardial derangements. Therefore, the protective action of D-*cis*- and L-*cis*-diltiazem may be due to their ability to inhibit the Na^+ channel. The above view can be supported by our previous findings that tetrodotoxin (Hara et al., 1998), lidocaine (Hara et al., 1993) and dilazep (Hara and Abiko, 1996), all of which have a Na^+ channel blocking action, are effective in attenuating the H_2O_2-induced mechanical dysfunction and decrease in the tissue levels of high-energy phosphates. It should be noted, however, that D-*cis*- and L-*cis*-diltiazem attenuated the H_2O_2-induced mechanical and metabolic derangements incompletely, even when they attenuated the H_2O_2-induced increase in $[Na^+]_i$ completely. It is possible therefore that the harmful action of H_2O_2 on the heart is not only due to activation of the fast Na^+ channel, but also due to other mechanisms, such as activation of nonselective cation channels (Jabr and Cole, 1995) and inactivation of creatine kinase (Banerjee et al., 1991).

According to Nasa et al. (1990), D-*cis*-diltiazem (15 μM) and L-*cis*-diltiazem (15 μM) are capable of attenuating the myocardial derangements induced by ischemia–reperfusion in the isolated, working rat heart. Therefore, the beneficial action of D-*cis*- and L-*cis*-diltiazem on the H_2O_2-induced changes may contribute to their protective effect on the myocardium against ischemia–reperfusion damage.

5. Conclusion

Both D-*cis*- and L-*cis*-diltiazem protect the myocardium against the H_2O_2-induced derangements in the isolated perfused rat heart. The protective action of D-*cis*- and L-*cis*-diltiazem may be due to inhibition of the H_2O_2-induced increase in $[Na^+]_i$, at least in part.

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