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Negative inotropic effect of endothelin-1 in the mouse right ventricle

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Abstract

Effects of endothelin-1 on the contraction and cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) of the mouse right ventricle were investigated. Endothelin-1 (1–300 nM) elicited a negative inotropic effect in a concentration-dependent manner. The endothelin-1-induced negative inotropy was antagonized by a selective endothelin ET_A receptor antagonist, BQ-123 (cyclo [Asp-Pro-Val-Leu-Trp-]; 3, 10 μM). Endothelin-1 reduced the peak amplitudes of both the $[Ca^{2+}]_i$ transient and contraction without changing inward Ca^{2+} current. The relationship between peak amplitude of $[Ca^{2+}]_i$ and peak force generated by changing the extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) was not affected by endothelin-1. In addition, the trajectory of the $[Ca^{2+}]_i$-contraction phase plane diagram obtained at 2 mM $[Ca^{2+}]_o$ in the absence of endothelin-1 was superimposable on that obtained at 4 mM $[Ca^{2+}]_o$ in the presence of endothelin-1 (300 nM). Endothelin-1 (300 nM) translocated protein kinase C from cytosol to membrane, suggesting activation of protein kinase C. Further, a selective protein kinase C inhibitor, bisindolylmaleimide I (10 μM), inhibited the endothelin-1-induced negative inotropy. These results suggest that endothelin-1 elicits negative inotropy by reducing the amplitude of the $[Ca^{2+}]_i$ transient without changing inward Ca^{2+} current through the activation of the endothelin ET_A receptor followed by protein kinase C activation in the mouse right ventricle.

Keywords: Endothelin-1; Cardiac muscle; $[Ca^{2+}]_i$ transient; Protein kinase C

1. Introduction

Over-expression or selective ''knock out'' of genes has become a useful tool to understand the role of a protein coded by a specific gene. In cardiac research, studies using transgenic mice (Luo et al., 1996; Kadambi et al., 1996; Kubota et al., 1997; Yao et al., 1998) are also increasing. However, few studies extant have focused on the physiological or pharmacological properties of cardiac muscle in the mouse (Nuss and Marban, 1994; Gao et al., 1998).

It is widely accepted that cardiac muscle contraction is modulated by several receptor agonists. Endothelin-1 has been shown to affect contraction of cardiac muscle. Many reports suggest that endothelin-1 elicits a positive inotropic effect in cardiac tissues (Ishikawa et al., 1988; Shah et al., 1989; Takanashi and Endoh, 1991) or cells (Qiu et al., 1992; Kramer et al., 1991; Moody et al., 1990; Moravec et al., 1989) in many species. Endothelin has been shown to activate phospholipase C and to increase phosphoinositide hydrolysis with resultant production of 1,2-diacylglycerol and inositol 1,4,5-trisphospate (IP_3) (Vigne et al., 1989; Takanashi and Endoh, 1992; Hilal-Dandan et al., 1992; Van Heugten et al., 1994). 1,2-diacylglycerol activates protein kinase C. Because a protein kinase C inhibitor will inhibit endothelin-1-induced positive inotropy, it is suggested that protein kinase C activation plays a central role in the action of endothelin-1 (Kramer et al., 1991; Hattori et al., 1993; Suzuki et al., 1998). However, in the mouse, modulation of the contraction of cardiac muscle by endothelin is not known.

The purpose of the present study was to examine the effect of endothelin-1 on the mouse right ventricle and to evaluate the mechanism of the effect of endothelin-1. We measured cytosolic $[Ca^{2+}]_i$ transients by using fura-2 and the force of isometric contraction simultaneously. In addition, protein kinase C activity and inward Ca^{2+} current were evaluated.

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2. Materials and methods

2.1. Preparation of muscle strips

Adult male mice (C57BL strain, 8–10 weeks, 18–23 g) were killed by dislocation of the cervical vertebrae. The heart was rapidly removed and washed in normal physiological salt solution (PSS) (mM: NaCl 145.0, KCl 5.0, $CaCl_2$ 2.0, $MgCl_2$ 1.0, glucose 10.0 and HEPES 10.0) at pH 7.4, saturated with 100% O_2 at room temperature. The right ventricular free wall was isolated, cut into strips 1 mm wide, 3–5 mm long and 0.4–0.6 mm thick, and placed in a 8-ml muscle bath containing PSS. The solution was bubbled constantly with 100% O_2 at room temperature.

2.2. Langendorff's method to load fura-2 into muscle strips

Changes in $[Ca^{2+}]_i$ were monitored with the fluorescent Ca^{2+} indicator, fura-2. To load fura-2 into the muscle strips the following procedure was adopted. Isolated hearts were perfused in the Langendorff's mode with PSS warmed to 30°C and at pH 7.4, gassed with 100% O_2. The coronary flow was normalized to 5 ml min^{-1}. After a 10 min equilibration period, the perfusion was switched to recirculating PSS (20 ml) containing 5 μM acetoxymethyl ester of fura-2 (fura-2/AM) and 0.025% cremophor EL, a noncytotoxic detergent, for 0.5–1 h. After the loading period, the perfusion was switched to fura-2-free PSS for 15 min to rinse the uncleaved fura-2/AM from the tissue.

2.3. Measurements of cytosolic Ca^{2+} concentration and muscle force

The force of contraction was recorded isometrically. One end of the muscle was fixed to the siliconized floor of the muscle bath and the other end was connected to a strain gauge transducer to monitor mechanical activity. Resting tension applied to each preparation was adjusted to the length at which the developed tension became maximal. During an equilibration period, the muscles were stimulated electrically by square pulses of 5 ms duration at 0.5 Hz with voltage 1.5-fold greater than that of the threshold intensity, using an electronic stimulator (Nihon Kohden, Tokyo, Japan). Fluorescence signals and contractile force were digitized by an A/D converter (A Burr-Brown, Tucson, AZ, USA) and an IBM PC/AT computer, at a sampling rate of 110 points s^{-1}. Fura-2 fluorescence was measured by the method of dual excitation. An excitation light, with a wavelength of 340 or 380 nm (alternated at 128 Hz) through two monochrometers in front of the UV-light source (75 W Xenon-lamp), was illuminated to the muscle. The ratio of the 500 nm fluorescence elicited by these two excitation wavelengths was calculated and used as an indicator of $[Ca^{2+}]_i$. The amplitudes of changes in the fura-2 signal or in isometric contraction were measured. Parameters obtained at a stimulation frequency of 0.5 Hz in the presence of 2.0 mM Ca^{2+} were used as a control (100%) unless otherwise mentioned. For the quantitative assessment of the signal, five successive twitch signals were averaged. Actual force developed by the muscle strips in the experiment in Sections 3.2 and 3.3 was 1.4 ± 0.3 mN ($n = 12$). Estimating the cross-sectional area of the ventricular strips to be 0.4–0.6 mm^2, tension development in muscles stimulated at 0.5 Hz in PSS with 2 mM Ca^{2+} was calculated to 2.8 mN/mm^2 cross-sectional area.

2.4. Measurement of protein kinase C activity

The whole right ventricular free wall was placed in the muscle bath containing normal PSS precisely like that used for measuring force of contraction. During an equilibration period, the muscle was stimulated at 0.5 Hz. After the muscle contraction became plateau, the muscle was treated with drug and was quickly frozen in liquid nitrogen and homogenized in 200 μl of an ice-cold buffer containing: tris(hydroxymethyl)aminomethane (Tris)–HCl (pH 7.4) 20 mM, sucrose 300 mM, ethylenediaminetetraacetic acid (EDTA) 5 mM, *O*,*O*′-bis(2-aminoethyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) 10 mM, phenylmethylsulphonyl fluoride 0.3 mM, leupeptin 25 μg ml^{-1} and 0.3% β-mercaptoethanol. The suspension was centrifuged at $105{,}000 \times g$ at 4°C for 60 min. The supernatant was used as a cytosolic fraction. The pellet was re-suspended in the same buffer containing 1% Triton X-100 and kept on ice for 30 min. The suspension treated with Triton X-100 was centrifuged again at 4°C for 60 min. The supernatant was used as a membrane fraction.

Protein kinase C activity was measured by a commercial protein kinase C assay system (RPN 77A, Amersham Japan, Tokyo, Japan). The cytosolic and membrane fractions were dissolved, respectively in a reaction medium containing: Tris–HCl (pH 7.5) 50 mM, calcium acetate 1 mM, magnesium acetate 15 mM, L α-phosphatidyl-L-serine 0.67 mol%, phorbol 12-myristate 13-acetate 2 μg ml^{-1}, synthetic peptide 75 μM, sodium azide 0.05% w/v and dithiothreitol 2.5 mM. The reaction was started by adding 50 μM ATP containing 0.2 μCi [γ-^{32}P]-ATP. After treatment at 37°C for 20 min, the reaction was stopped by 300 mM orthophosphoric acid containing carmosine red. The radioactivity in the synthetic peptide was evaluated with a liquid scintillation spectrometer.

2.5. Measurement of inward Ca^{2+} currents

Ventricular myocytes were isolated enzymatically using a Langendorff's perfusion procedure. Isolated heart was perfused with nominally Ca^{2+}-free PSS containing bovine serum albumin (1 mg/ml) warmed to 37°C. All solutions were bubbled with 100% O_2. The coronary flow was normalized to 2–3 ml min^{-1}. After 10 min, the perfusion

was switched to recirculating 30 μM Ca^{2+} PSS (25 ml) containing collagenase (1 mg/ml; Wako pure chemicals, Tokyo, Japan), protease type XIV (0.2 mg/ml; Sigma, St. Louis, MO, USA) and bovine serum albumin (1 mg/ml) for 20–25 min. Then the perfusion was switched to PSS with 100 μM Ca^{2+} for 10 min to rinse the collagenase containing solution. At the end of the perfusion period, the heart was transferred into a dish containing PSS with 100 μM Ca^{2+}. Ventricle was cut into small pieces and gently triturated for 5 min. After this procedure, the cells were stored at room temperature for an hour.

Inward Ca^{2+} currents were recorded in whole-cell, voltage-clamp configuration of the patch-clamp technique. The patch electrodes, made of borosilicate glass capillaries, were fire polished to have a resistance of 2–5 MΩ when filled with the pipette solution (120 mM CsCl, 20 mM tetraethylammonium chloride, 14 mM EGTA, 5 mM Mg–ATP, 5 mM Na–creatine phosphate, 0.2 mM Na–GTP and 10 mM HEPES; pH 7.3 with CsOH). The electrodes were connected to an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA), and DigiData 1200 (Axon Instruments) interface controlled by pClamp 6.0.4 software (Axon Instruments) was used to generate command pulse and acquire data.

Cells were perfused with normal PSS with 2 mM Ca^{2+}. We used a holding potential of −60 mV. K^+ currents were suppressed by internal Cs^+ and tetraethylammonium. To suppress Na^+ currents, 100 ms step pulse to −40 mV was added before the application of 300 ms test potential. These depolarizing pulses were added every 10 s. Difference between peak and the value at the end of the depolarizing pulse was detected as inward current. Inward currents developed in this procedure were completely inhibited by 10 μM nimodipine (data not shown) suggests that these currents consisted of L-type Ca^{2+} current. All the experiment was done under room temperature and we used the data in which no rundown of L-type Ca^{2+} current was observed during the experimental period (< 30 min).

2.6. Chemicals

Endothelin-1, sarafotoxin S6c, IRL 1620 (succinyl-[Glu^9, $Ala^{11,15}$]endothelin-1_{8-21}) and leupeptin were purchased from the Peptide Institute (Osaka, Japan). BQ-123 (cyclo [Asp-Pro-Val-Leu-Trp-]) was kindly donated by Dr. T. Okada (Ciba-Geigy, Takarazuka, Japan). Other chemicals used were fura-2/AM (Wako), phorbol 12,13-dibutylate (PDB) (Sigma), bisindolylmaleimide I (Calbiochem, La Jolla, CA, USA), cremophor EL (Nacalai Tesque, Kyoto, Japan). BQ-123, fura-2/AM and bisindolylmaleimide I were dissolved in dimethyl sulfoxide. Endothelin-1 and sarafotoxin S6c were dissolved in dilute acetic acid (0.1%). IRL-1620 was dissolved in 0.01 N NaOH. The final concentration of the solvent was less than 0.3% which, alone, had no effect on $[Ca^{2+}]_i$ or contractile force.

2.7. Statistics

The numerical data were expressed as mean ± standard error (S.E.). Differences between mean values were evaluated by Student's *t*-test and a probability of less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of endothelin-1

Fig. 1 shows the concentration–response curve for the inotropic effect of endothelin-1 on the mouse right ventricle in the presence or absence of the selective endothelin ET_A receptor antagonist, BQ-123 (3 and 10 μM). Endothelin-1 elicited a negative inotropic effect in a concentration-dependent manner with a threshold concentration of 10 nM. The onset of the response was slow (taking about 3 min), and 10–20 min was required for a plateau to be reached. The selective endothelin ET_B receptor agonists, sarafotoxin S6c (100, 300 nM) and IRL 1620 (300 nM), in contrast, had no effect on the contractile force (to 103.6 ± 4.1% $n = 4$, 106.0 ± 3.1% $n = 4$ and 97.7 ± 6.1% $n = 4$, respectively). Addition of BQ-123 (3 and 10 μM) did not affect the basal force (to 104.3 ± 3.2%, $n = 12$ and to 113.7 ± 8.2%, $n = 6$, respectively). BQ-123 (3 μM) significantly shifted the concentration–response curve for endothelin-1 to the right ($n = 4$) and BQ-123 (10 μM) completely inhibited the negative inotropic effect of endothelin-1 (300 nM) ($n = 6$).

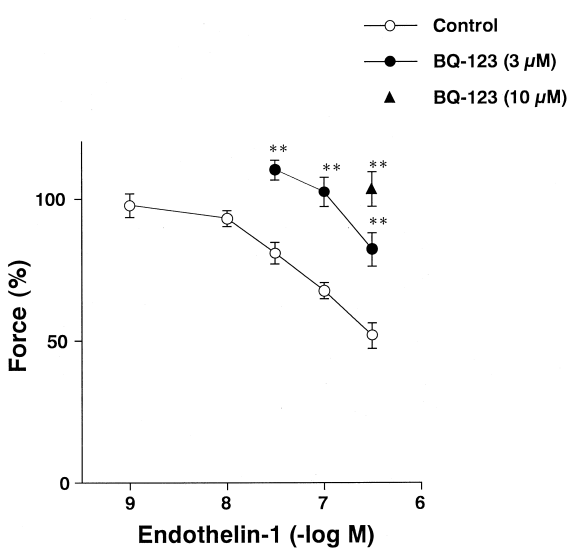

Fig. 1. Concentration–response curves for the negative inotropic effects of endothelin-1 in mouse right ventricle in the absence ($n = 5–9$) or presence ($n = 4–6$) of BQ-123 (3 or 10 μM). One concentration of endothelin-1 was added to each preparation. Value obtained before addition of endothelin-1 was taken as 100%. Each point indicates mean peak contractile force. Vertical lines indicate S.E. ** Significantly different from control ($P < 0.01$).

3.2. Effects of endothelin-1 on muscle contraction and $[Ca^{2+}]_i$ transients

Simultaneous measurement of muscle contraction and $[Ca^{2+}]_i$ transient showed that endothelin-1 did not affect the diastolic levels of both force and the $[Ca^{2+}]_i$ transient (data not shown, $n = 6$). Fig. 2 shows representative tracings for the muscle contraction (upper) and the $[Ca^{2+}]_i$ transient (lower) in the presence or absence of endothelin-1 (300 nM). Endothelin-1 (300 nM) decreased the peak contractile force and the peak $[Ca^{2+}]_i$ transient to $34.2 \pm 4.2\%$ and $72.4 \pm 3.7\%$ ($n = 6$) of control, respectively (Fig. 2, Table 1). Inhibition of force by endothelin-1 was slightly stronger in fura-2-loaded muscle compared to that in the muscle without fura-2-loading. We did not examine the reason for this difference. Table 1 shows the effect of endothelin-1 (300 nM) on contractile parameters. The maximum rate of rise of both force (dF/dt max) and $[Ca^{2+}]_i$ transient (dR/dt max) were also decreased by endothelin-1 and the amplitude of inhibition of these parameters were comparable to that of peak values (Table 1). Endothelin-1 did not change the time to peak force and the time to half relaxation (Table 1). In the experiment illustrated in Fig. 2, the time to peak of $[Ca^{2+}]_i$ transient is slightly decreased in the presence of endothelin-1, however, the mean value of the time to peak $[Ca^{2+}]_i$ was not affected by endothelin-1 (Table 1). The time to half decay of $[Ca^{2+}]_i$ was not affected by endothelin-1. We also estimated the time constants of $[Ca^{2+}]_i$ decline for each $[Ca^{2+}]_i$ transient by using a single exponential decline. Endothelin-1 did not change the time constant of $[Ca^{2+}]_i$ decline.

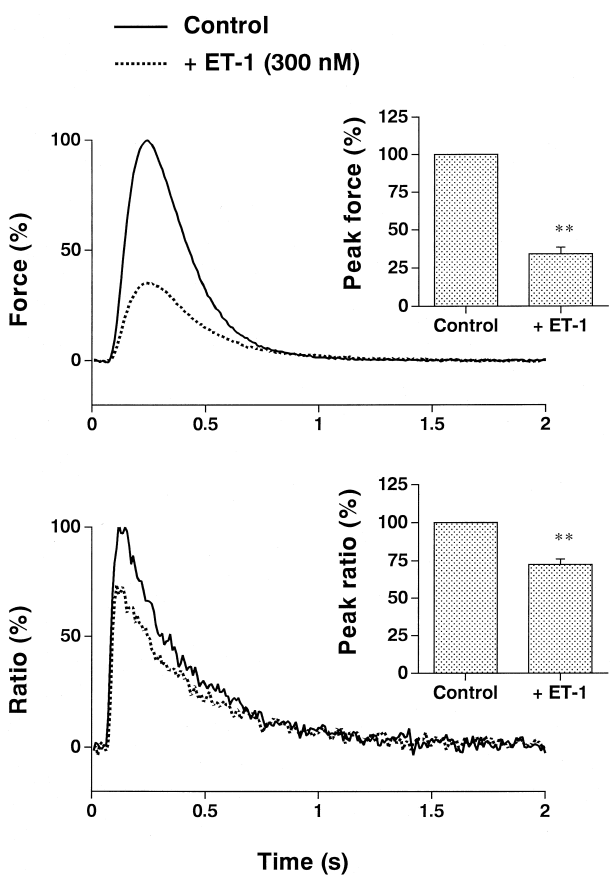

Fig. 2. Averaged tracings of five successive $[Ca^{2+}]_i$ transients and isometric contractions in the absence or presence of endothelin-1 (ET-1; 300 nM) in the mouse right ventricle loaded with fura-2. Value obtained in the absence of endothelin-1 was taken as 100%. Stimulation was applied at time zero. Insets show the statistical results. Each column indicates mean contractile force or the peak amplitude of $[Ca^{2+}]_i$ transient ($n = 6$). Vertical lines indicate S.E. ** Significantly different from control ($P < 0.01$).

Table 1
Effects of endothelin-1 (300 nM) on contractile parameters
Values are means ± S.E. ($n = 6$).

	Control	Endothelin-1
Peak force (%)	100	34.2 ± 4.2[a]
dF/dt max (%)	100	37.3 ± 4.3[a]
Time to peak force (ms)	195.5 ± 5.1	192.4 ± 6.4
Time to half relaxation (ms)	175.8 ± 20.4	231.8 ± 32.1
Peak $[Ca^{2+}]_i$ transient (%)	100	72.4 ± 3.7[a]
dR/dt max (%)	100	71.8 ± 7.5[a]
Time to peak $[Ca^{2+}]_i$ (ms)	89.4 ± 2.8	87.9 ± 1.9
Time to half decay (ms)	202.3 ± 12.5	208.3 ± 14.3
Time constant of $[Ca^{2+}]_i$ decline (ms)	298.0 ± 15.2	336.1 ± 30.5

[a] $P < 0.01$ as compared with Control.

3.3. Effects of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) on muscle contraction and $[Ca^{2+}]_i$ transients

Decrement of $[Ca^{2+}]_o$ from 2 to 1 mM did not affect the diastolic levels of both force and the $[Ca^{2+}]_i$ transient (data not shown, $n = 6$). Fig. 3 shows representative tracings for the muscle contraction (upper) and the $[Ca^{2+}]_i$ transient (lower) at 2 mM $[Ca^{2+}]_o$ or 1 mM $[Ca^{2+}]_o$. Decrement of $[Ca^{2+}]_o$ decreased the peak contractile force and the peak $[Ca^{2+}]_i$ transient to $40.9 \pm 5.0\%$ and $76.8 \pm 1.9\%$ ($n = 6$) of control, respectively (Fig. 3, Table 2). Table 2 shows the effect of $[Ca^{2+}]_o$ on the contractile parameters. dF/dt max and dR/dt max were also decreased by decrement of $[Ca^{2+}]_o$ and inhibition of these parameters were comparable to that of peak values. Decrement of $[Ca^{2+}]_o$ did not change the time to peak force and the time to half relaxation. The time to peak $[Ca^{2+}]_i$ was not affected by decrement of $[Ca^{2+}]_o$. Both the time constant of $[Ca^{2+}]_i$ decline and the time to half decay of $[Ca^{2+}]_i$ were increased, although the latter did not reach statistical significance (Table 2).

3.4. Effects of endothelin-1 on Ca^{2+} responsiveness of myofilaments

To evaluate the relationship between $[Ca^{2+}]_i$ transients and force development quantitatively, we first compared the relationship between the peak amplitude of the $[Ca^{2+}]_i$

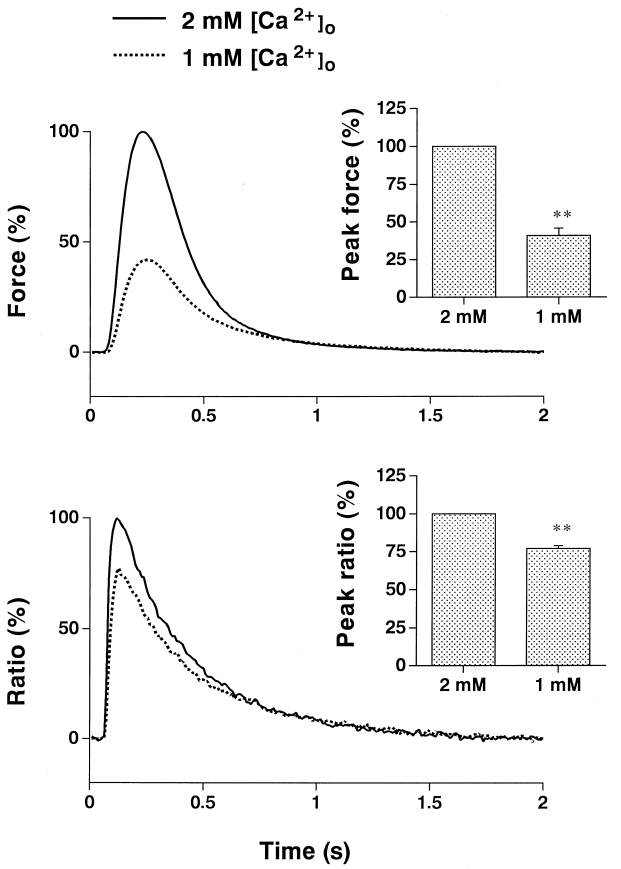

Fig. 3. Averaged tracings of five successive $[Ca^{2+}]_i$ transients and isometric contractions at 2 mM $[Ca^{2+}]_o$ or 1 mM $[Ca^{2+}]_o$ in the mouse right ventricle loaded with fura-2. Value obtained at 2 mM $[Ca^{2+}]_o$ was taken as 100%. Stimulation was applied at time zero. Insets show the statistical results. Each column indicates mean contractile force or the peak amplitude of $[Ca^{2+}]_i$ transient ($n = 6$). Vertical lines indicate S.E. ** Significantly different from control ($P < 0.01$).

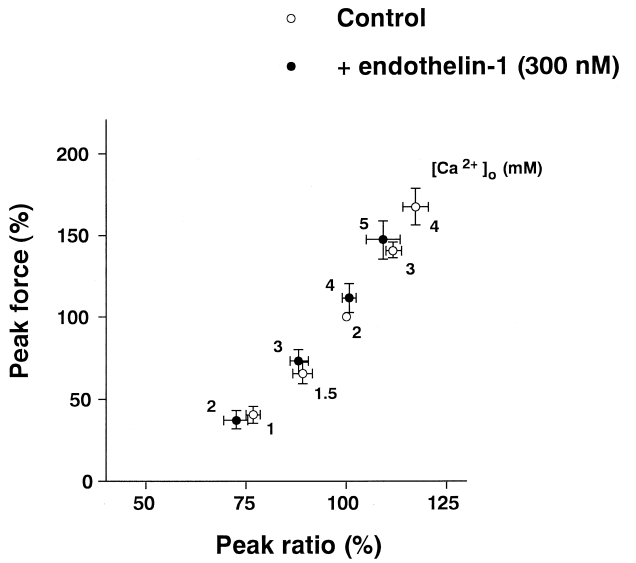

Fig. 4. Relationship between peak amplitude of $[Ca^{2+}]_i$ transient and peak force obtained in mouse right ventricle by changing external $[Ca^{2+}]_o$ concentration in the absence ($n = 6$) or presence ($n = 6$) of endothelin-1 (300 nM). Peak force and peak amplitude of $[Ca^{2+}]_i$ transient (peak ratio) obtained in normal PSS were taken as 100%. Each point represents mean of the each value. Vertical lines indicate S.E. External Ca^{2+} concentration was changed from 1 to 1.5, 2, 3 and 4 mM under control condition and from 2 to 3, 4 and 5 mM in the presence of endothelin-1.

transient and the peak force generated by varying $[Ca^{2+}]_o$ in the presence and absence of endothelin-1 (300 nM) (Fig. 4). With stepwise increases in $[Ca^{2+}]_o$ from 1 to 4 mM, the amplitude of force increased from $40.9 \pm 5.0\%$ to $166.7 \pm 11.2\%$ ($n = 6$) in a concentration-dependent manner. The peak amplitude of the $[Ca^{2+}]_i$ transient was also increased from $76.8 \pm 1.9\%$ to $117.4 \pm 3.3\%$. In the presence of endothelin-1 (300 nM), the stepwise increases in $[Ca^{2+}]_o$ from 2 mM to 5 mM increased the amplitude of force from $37.8 \pm 5.7\%$ to $146.7 \pm 11.8\%$ ($n = 6$) in a concentration-dependent manner. The peak amplitude of the $[Ca^{2+}]_i$ transient was also increased from $72.4 \pm 3.1\%$ to $109.3 \pm 4.2\%$. Values for $[Ca^{2+}]_i$ and force, plotted in Fig. 4, indicate that endothelin-1 did not change the $[Ca^{2+}]_i$-force relationship.

To further clarify the effect of endothelin-1 on myofilament Ca^{2+} responsiveness, the trajectory of the $[Ca^{2+}]_i$-force relationship in the presence of 300 nM endothelin-1 at 4 mM $[Ca^{2+}]_o$ was compared with that of the control (at 2 mM $[Ca^{2+}]_o$). Fig. 5 shows the representative trajectory. The curves overlapped. Similar results were obtained in other muscle preparations ($n = 6$).

Table 2
Effects of $[Ca^{2+}]_o$ on contractile parameters
Values are means ± S.E. ($n = 6$).

	Control (2 mM)	1 mM
Peak force (%)	100	40.9 ± 5.0[a]
dF/dt max (%)	100	41.7 ± 5.2[a]
Time to peak force (ms)	203.0 ± 8.4	204.5 ± 9.0
Time to half relaxation (ms)	219.7 ± 16.3	251.5 ± 23.1
Peak $[Ca^{2+}]_i$ transient (%)	100	76.8 ± 1.9[a]
dR/dt max (%)	100	75.9 ± 4.2[a]
Time to peak $[Ca^{2+}]_i$ (ms)	81.8 ± 3.3	81.8 ± 2.3
Time to half decay (ms)	215.9 ± 12.9	243.2 ± 15.5
Time constant of $[Ca^{2+}]_i$ decline (ms)	326.6 ± 22.3	381.8 ± 23.9[b]

[a] $P < 0.01$ as compared with Control.
[b] $P < 0.05$ as compared with Control.

3.5. Effect of a protein kinase C inhibitor on the endothelin-1-induced negative inotropic effect

To clarify whether protein kinase C activation is involved in the negative inotropic effect of endothelin-1, we examined the effect of a selective protein kinase C inhibitor, bisindolylmaleimide I (10 μM), on the action of endothelin-1 (300 nM). Fig. 6A shows the effect of endothelin-1 (300 nM), the protein kinase C activator, PDB (10 nM), and bisindolylmaleimide I (10 μM) itself. En-

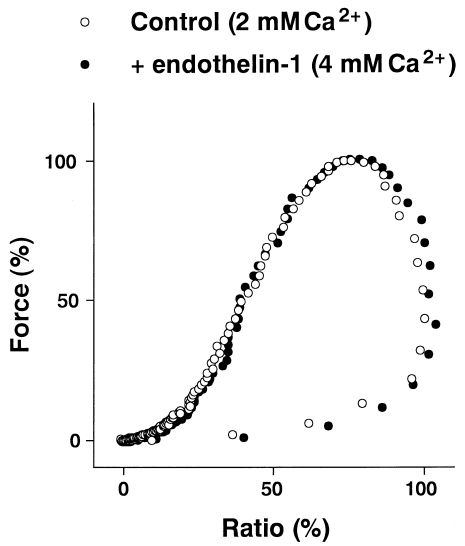

Fig. 5. The phase-plane diagram converted from the time-dependent changes in averaged signals of isometric force and fura-2 fluorescence ratio in the absence ($[Ca^{2+}]_o$; 2 mM) or presence of endothelin-1 (300 nM) ($[Ca^{2+}]_o$; 4 mM). Value obtained in normal PSS was taken as 100%.

dothelin-1 decreased the force to $51.7 \pm 4.6\%$ ($n = 8$). PDB also induced a negative inotropic action approximate to that of endothelin-1 ($46.1 \pm 2.9\%$, $n = 4$). On the contrary, bisindolylmaleimide I alone gradually increased the force to $173.5 \pm 19.0\%$ ($n = 9$), reaching a plateau 40–60 min after addition of bisindolylmaleimide I. In the presence of bisindolylmaleimide I (10 μM), endothelin-1 (300 nM) and PDB (10 nM) no longer elicited a negative inotropic effect ($116.2 \pm 8.9\%$, $n = 9$; and 93.3 ± 5.8, $n = 5$) (Fig. 6B).

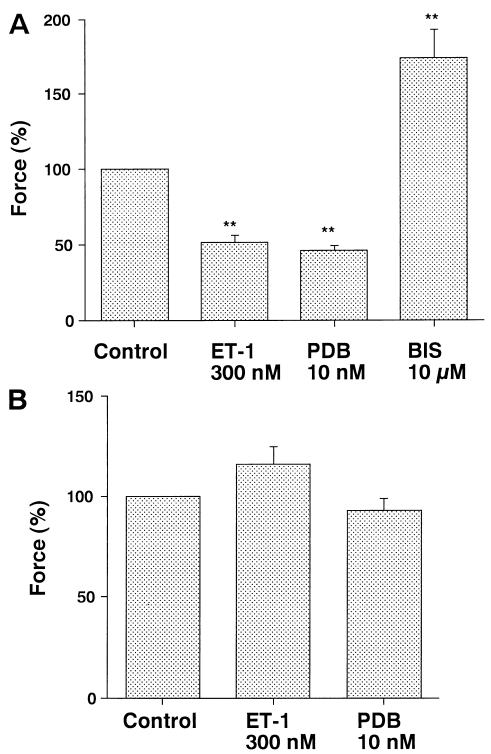

Fig. 6. (A) Effects of endothelin-1 (ET-1; 300 nM, $n = 8$), PDB (10 nM, $n = 4$) and bisindolylmaleimide I (BIS; 10 μM, $n = 9$). Value obtained before addition of drug was taken as 100%. (B) Effects of endothelin-1 (ET-1; 300 nM, $n = 9$), PDB (10 nM, $n = 5$) in the presence of bisindolylmaleimide I (10 μM). Value obtained before addition of endothelin-1 or PDB was taken as 100%. Each column indicates mean of the percentage of the contractile force. Vertical lines indicate S.E. ** Significantly different from control ($P < 0.01$).

3.6. Effect of endothelin-1 on the membrane protein kinase C activity

Fig. 7 shows the effect of endothelin-1 (300 nM) on the membrane protein kinase C activity. Treatment of the muscle with endothelin-1 for 6 min increased membrane protein kinase C activity to about 169% (from 7.0 ± 0.6 pmol min^{-1} mg^{-1} protein to 11.8 ± 1.8 pmol min^{-1} mg^{-1} protein). However, endothelin-1 did not significantly change the cytosolic protein kinase C activity.

3.7. Effect of endothelin-1 on inward Ca^{2+} currents

The effects of endothelin-1 (300 nM) on inward Ca^{2+} current were examined in single mouse ventricular myocyte (Fig. 8). Fig. 8A shows representative tracings of inward Ca^{2+} current in the absence and presence of 300 nM endothelin-1. Fig. 8B shows the current–voltage relationship before and after exposure to endothelin-1 (300 nM). Mean peak inward Ca^{2+} current activated at 0 mV in the absence and presence of endothelin-1 was 1011.1 ± 168.2 pA ($n = 6$) and. 1050.1 ± 183.5 pA ($n = 6$), respectively. When peak inward Ca^{2+} current activated at 0 mV before addition of endothelin-1 was normalized as 100%,

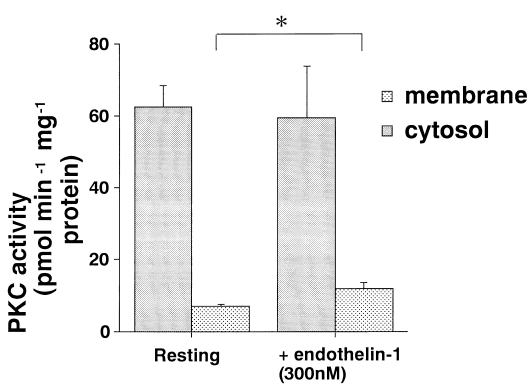

Fig. 7. Effects of endothelin-1 (300 nM) on membrane protein kinase C activity in the mouse right ventricle. Muscle strips were incubated with endothelin-1 for 6 min. Protein kinase C activity was shown by pmol ATP incorporated into synthetic peptide per min per mg of cardiac muscle protein (pmol min^{-1} mg^{-1} protein). Each column represents mean of four experiments and S.E. is shown by vertical bar. * Significantly different from the value in resting muscle ($P < 0.05$).

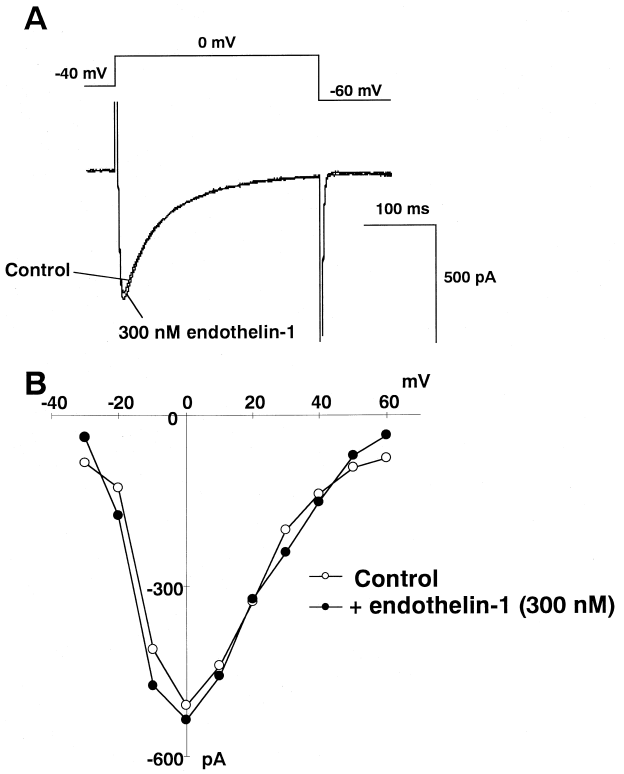

Fig. 8. (A) Typical tracings of inward Ca^{2+} current in the absence or presence of endothelin-1 (300 nM) in the mouse ventricular myocyte. (B) Current–voltage relationship before and after exposure to endothelin-1 (300 nM).

peak inward Ca^{2+} current in the presence of endothelin-1 was 103.2 ± 3.1%. These results suggest that endothelin-1 did not affect the amplitude of inward Ca^{2+} current.

4. Discussion

In the present study, we demonstrated that endothelin-1 elicited a negative inotropic effect in the mouse right ventricle. A selective endothelin ET_A receptor antagonist, BQ-123, inhibited the negative inotropic effect of endothelin-1. In contrast, the selective endothelin ET_B receptor agonists, sarafotoxin S6c and IRL 1620, did not affect the twitch contractile force. These results suggest that the negative inotropic effect of endothelin-1 is mediated through the endothelin ET_A receptor in the mouse right ventricle.

It has been shown that endothelin-1 activates phospholipase C to increase production of 1,2-diacylglycerol and inositol 1,4,5-trisphospate (IP_3) (Vigne et al., 1989; Takanashi and Endoh, 1992; Hilal-Dandan et al., 1992; Van Heugten et al., 1994). 1,2-Diacylglycerol is known to activate protein kinase C. In the present study, we confirmed that endothelin-1 increased the protein kinase C activity of a membrane fraction. We further examined whether protein kinase C activation is involved in the negative inotropic effect of endothelin-1 in the mouse right ventricle by using a protein kinase C inhibitor, bisindolylmaleimide I. Results indicated that endothelin-1 failed to elicit a negative inotropic effect in the presence of bisindolylmaleimide I. In addition, a protein kinase C activator, PDB, also elicited a negative inotropic effect that was eradicated by bisindolylmaleimide I. Although bisindolylmaleimide I has a high selectivity for protein kinase C, bisindolylmaleimide I has been shown to inhibit other protein kinases, such as protein kinase A at a high concentration (Toullec et al., 1991). We used a bisindolylmaleimide I concentration of 10 μM; in an in vitro study, this concentration of bisindolylmaleimide I was sufficient to inhibit the other kinases. However, 1 μM bisindolylmaleimide I did not inhibit the negative inotropic effect of PDB (data not shown), which suggests that the concentration of bisindolylmaleimide I inside the cell was far lower than that outside the cell. Bisindolylmaleimide I applied alone demonstrated an increase in the force. Nicolas et al. (1998) reported that protein kinase C inhibitors such as staurosporine, calphostin C and bisindolylmaleimide I increase the amplitude of the $[Ca^{2+}]_i$ transient and contraction in rat ventricular myocytes. Further, they found that PDB elicits a negative inotropic effect. These findings, taken together, make it likely that bisindolylmaleimide I elicits its action by inhibition of endogenous protein kinase C activity.

We also found that endothelin-1 decreased the amplitude of the $[Ca^{2+}]_i$ transient during twitch contraction. There are at least two possibilities for the reduction of the $[Ca^{2+}]_i$ transient. First, endothelin-1 may reduce the Ca^{2+} influx. Consistent with this possibility, it was reported in various studies using the patch-clamp technique — e.g., in guinea-pig ventricular myocyte (Tohse et al., 1990), guinea-pig atrial myocyte (Ono et al., 1994), human cardiac myocyte (Cheng et al., 1995) and rabbit ventricular myocyte (Kelso et al., 1998) — that endothelin-1 decreases the inward Ca^{2+} current. Conversely, it has also been reported that endothelin-1 *increases* inward Ca^{2+} current in guinea-pig ventricular myocyte (Tong et al., 1995) and rabbit cardiac myocyte (Lauer et al., 1992). However, there has been no report for the effect of endothelin-1 on the inward Ca^{2+} current in mouse ventricular myocyte. We measured the L-type Ca^{2+} current in mouse ventricular myocyte by using patch-clamp technique and found that endothelin-1 did not change inward Ca^{2+} current. This result suggests that direct inhibition of Ca^{2+} influx may not be involved in the reduction of the $[Ca^{2+}]_i$ transient in the mouse right ventricular muscle.

The second possibility is that decrease in the amount of Ca^{2+} release for a given Ca^{2+} trigger may decrease the amplitude of the $[Ca^{2+}]_i$ transient. If the amount of Ca^{2+} pooled in the sarcoplasmic reticulum would have reduced, the amount of Ca^{2+} release for a given Ca^{2+} trigger might be decreased. Rogers et al. (1990) showed that protein kinase C activation by phorbol ester decreases Ca^{2+} accu-

mulation into the sarcoplasmic reticulum. In order to test this possibility, we measured the time constant of $[Ca^{2+}]_i$ decline to test whether Ca^{2+} pump on the sarcoplasmic reticulum was inhibited by endothelin-1. The results suggest that, in the presence of endothelin-1, the time constant of $[Ca^{2+}]_i$ decline tended to be increased but not significant, suggesting that inhibition of Ca^{2+} pump on the sarcoplasmic reticulum may not be involved in the inhibition of the $[Ca^{2+}]_i$ transient by endothelin-1. Another possible mechanism to decrease the amount of Ca^{2+} in the sarcoplasmic reticulum is to stimulate the leak of Ca^{2+} from the sarcoplasmic reticulum. Tohse et al. (1990) showed that endothelin-1 decreased the inward Ca^{2+} current but it was abolished in the presence of ryanodine, suggesting that endothelin-1 increases the leakage of Ca^{2+} from the sarcoplasmic reticulum resulted in an inhibition of inward Ca^{2+} current by Ca^{2+}-induced inactivation. Furthermore, there may be a mechanism, which affects the amount of Ca^{2+} released from the sarcoplasmic reticulum, without changing Ca^{2+} load in the sarcoplasmic reticulum. Further study is necessary to clarify the effect of endothelin-1 on Ca^{2+} release function.

Decrease in the Ca^{2+} responsiveness of myofilaments is also a possible mechanism to reduce twitch contraction. However, endothelin-1 did not change the relationship between the peak amplitude of the $[Ca^{2+}]_i$ transient and the peak force obtained by varying $[Ca^{2+}]_o$ (Fig. 4). In addition, the trajectory of the $[Ca^{2+}]_i$-force relationship in the presence of 300 nM endothelin-1 at 4 mM $[Ca^{2+}]_o$ was similar to that of the control (at 2 mM $[Ca^{2+}]_o$) (Fig. 5). These results suggest that the negative inotropic effect of endothelin-1 is not mediated by decrease in Ca^{2+} responsiveness of myofilaments.

In the present study, we demonstrate that endothelin-1 decreased the $[Ca^{2+}]_i$ transient, resulting in a negative inotropic effect in the mouse right ventricle. In other species, however, such as guinea pig, rabbit, ferret, rat and human (Ishikawa et al., 1988; Hattori et al., 1993; Moravec et al., 1989; Moody et al., 1990; Qiu et al., 1992; Shah et al., 1989; Takanashi and Endoh, 1991), endothelin-1 elicits a positive inotropic effect by increasing the amplitude of the $[Ca^{2+}]_i$ transient and/or the Ca^{2+} responsiveness of myofilaments. Similar differences among species are observed with regard to the α-adrenergic stimulation; α-adrenergic stimulation has been shown to elicits positive inotropic effect in the cardiac preparations such as papillary muscles, ventricular strips and isolated cardiomyocytes from several species such as rabbit, guinea pig, cat, dog and human (for review see Endoh, 1986; Terzic et al., 1993). On the other hand, the effect of α-adrenergic stimulation in rat cardiac muscle is controversial; α-adrenergic stimulation has been shown to have both positive (Otani et al., 1988; Fedida and Bouchard, 1992) and negative inotropic (Kissling et al., 1997) effects. On the other hand, in the adult mouse myocardia, sustained negative inotropic effect mediated by α-adrenoceptors is reported (Tanaka et al., 1995). The explanation for such differences is currently unclear. One possibility is that phospholipase C activation may affect multiple processes of EC-coupling (Ca^{2+} influx, Ca^{2+} responsiveness and Ca^{2+} release from the sarcoplasmic reticulum), and each individual process may contribute variably among animal species and under different experimental conditions. The balance of these multiple effects may limit cardiac response to agonists that activate phospholipase C. The present study was conducted at room temperature. However, the negative inotropic effect of endothelin-1 in mouse ventricle was evident also at 37°C (data not shown). Thus, it is unlikely that the temperature determine whether endothelin-1 induces positive or negative inotropic effect.

In conclusion, endothelin-1 elicited a negative inotropic effect in the mouse right ventricle via activation of endothelin ET_A receptors. The negative inotropic effect was caused by reduction of the $[Ca^{2+}]_i$ transient without changing inward Ca^{2+} current, and endothelin-1 demonstrated no effect on Ca^{2+} responsiveness of myofilaments. The protein kinase C pathway is involved in these mechanisms.

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