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Positive inotropic effect of endothelin-1 in the neonatal mouse right ventricle

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Abstract

In neonatal mouse right ventricles, endothelin-1 (ET-1, 1–300 nM) induced a dose-dependent increase in twitch contractions and the dose–response curve was shifted to the right by BQ-123 (10 μM), an endothelin ET_A receptor antagonist. The ET-1 (100 nM)-induced positive inotropy was accompanied by an increase in $[Ca^{2+}]_i$ transients without any change in the $[Ca^{2+}]_i$–force relationship. Ryanodine (1 μM) partially decreased the $[Ca^{2+}]_i$ transients and contractile force, but did not affect the ET-1 (100 nM)-induced positive inotropy. Reduction of $[Na^+]_o$ elicited an increase in contractile force, and this effect was significantly inhibited by KB-R7943 (30 μM), an inhibitor of the Na^+–Ca^{2+} exchanger. KB-R7943 (30 μM) almost completely suppressed the positive inotropic effect of ET-1. Activation of protein kinase C (PKC) by phorbol 12,13-dibutylate (100 nM) decreased the contractile force, an effect which was suppressed by bisindolylmaleimide I (3 μM). On the other hand, the ET-1-induced positive inotropic effect was unaffected by bisindolylmaleimide I (3 μM). These results suggest that the positive inotropic effect of ET-1 in neonatal mouse right ventricles is caused by the increase in $[Ca^{2+}]_i$ transients through activation of the endothelin ET_A receptor and the increase in Ca^{2+} influx via the Na^+–Ca^{2+} exchanger during an action potential. Furthermore, the ET-1-induced positive inotropy is independent of the effects of PKC, which makes it distinct from the ET-1-mediated pathways reported for cardiac tissues in other species.

Keywords: Endothelin-1; Cardiac muscle; Neonate, mouse; Na^+–Ca^{2+} exchanger; $[Ca^{2+}]_i$ transient

1. Introduction

Endothelin-1 (ET-1) increases contractile force in several cardiac tissues (Ishikawa et al., 1988; Takanashi and Endoh, 1991) and in cardiomyocytes (Moravec et al., 1989; Qiu et al., 1992) isolated from various species. The positive inotropic effect of ET-1 is thought to be mediated by the activation of phosphoinositide hydrolysis (Vigne et al., 1989; Hilal-Dandan et al., 1992; Krämer et al., 1991) and the subsequent activation of protein kinase C (PKC) (Hattori et al., 1993; Suzuki et al., 1998). PKC may activate Na^+–H^+ exchangers to induce cytoplasmic alkalosis and Ca^{2+} sensitization of contractile elements (Krämer et al., 1991; Woo and Lee, 1999; Russell and Molenaar, 2000) or to induce accumulation of Na^+ in the cytoplasm and thereby activate the Ca^{2+} influx mode of Na^+–Ca^{2+} exchangers (Ballard and Schaffer, 1996; Yang et al., 1999). Among laboratory-animal species, mice are the most frequently used in studies employing overexpression or knock-out gene technology. For mouse cardiac tissues, ET-1 has been reported to elicit negative inotropy in adult cardiac muscle but positive inotropic effects in neonatal tissue (Sekine et al., 1999).

On the other hand, many reports have suggested that ET-1 modulates various physiological and pathophysiological responses in the heart in various species, and, furthermore, the inotropic effects of ET-1 on the myocardium may change under various conditions, including developmental stages or pathophysiological conditions (Khandoudi et al., 1994; Sekine et al., 1999; Wölkart et al., 2000), indicating

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the complexity and importance of the mechanism of the ET-1-induced effects on cardiac tissues. We recently determined that the ET-1-induced negative inotropy in adult mouse (8–10 weeks) right ventricles is mediated by the activation of PKC and the subsequent decrease in $[Ca^{2+}]_i$ transients (Izumi et al., 2000). However, the mechanism of the positive inotropic effect of ET-1 in the neonatal mouse heart has not yet been clarified. In the present work, aiming to evaluate the mechanism of the positive inotropic effect of ET-1 in neonatal mouse right ventricles, we measured contractile force and, at the same time, monitored changes in the number of $[Ca^{2+}]_i$ transients using fura-2, a fluorescent Ca^{2+} indicator.

2. Methods

2.1. Preparation of muscle strips

CD-1 neonatal mice (Charles River Japan, Kanagawa, Japan) were killed by decapitation at 1 or 2 days of age. The hearts were 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; pH 7.4) saturated with 100% O_2 at room temperature. The right ventricular free wall was isolated and placed in 8 ml of muscle bath containing PSS. The solution was bubbled constantly with 100% O_2 at room temperature (25 °C). Animal care and treatment were in conformity with the institutional guidelines of the University of Tokyo and are consistent with the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institute of Health.

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

Contractile force was measured and, at the same time, changes in $[Ca^{2+}]_i$ were monitored using fura-2, a fluorescent Ca^{2+} indicator. The monitoring of $[Ca^{2+}]_i$ using fura-2 was performed as described by Miyamoto et al. (1999) and Izumi et al. (2000). Briefly, each isolated right ventricular free wall (approximately 2 mm in length and 1.2 mm in width) of 1- to 2-day-old neonates was placed in a fura-2 solution (PSS containing 50 μM fura-2/AM and 0.025% cremophor EL, a non-cytotoxic detergent) at 33 °C for 1–1.5 h. After the loading period, the preparations were washed in normal PSS to rinse the uncleaved fura-2/AM from the tissue.

The force of contraction was recorded isometrically as previously described (Izumi et al., 2000). A muscle strip was held horizontally in a temperature-controlled bath. 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. The bath was filled with normal PSS, and the resting tension applied to each preparation was adjusted to 2 mN. During the equilibration period, an electronic stimulator (Nihon Kohden, Tokyo, Japan) was used to stimulate the muscles electrically using square pulses of 5 ms duration at 0.5 Hz with a voltage 1.5-fold greater than the threshold intensity (usual voltage used was 75 mV). Fluorescence signals and contractile force were digitized by an A/D converter (A Burr-Brown, Tucson, AZ) 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. The muscle was illuminated by 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). The ratio of the 500 nm fluorescence elicited by these two excitation wavelengths was calculated and used as an indicator of $[Ca^{2+}]_i$. We measured the amplitudes of changes in the fura-2 signal or in isometric contraction. 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, 30 successive twitch signals were averaged.

NaCl was replaced with an equivalent amount of LiCl in low Na^+ PSS.

2.3. Chemicals

ET-1 and sarafotoxin S6c were purchased from the Peptide Institute (Osaka, Japan). Other chemicals used were ouabain, acetoxymethyl ester fura-2 (fura-2/AM; Wako, Osaka, Japan), phorbol 12,13-dibutylate (PDB), ryanodine, bay K8644 (Sigma, St. Louis, MO), BQ-123 [cyclo (-D-Trp-D-Asp-Pro-D-Val-Leu-)Na^+], BQ-788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-gamma-methylleucyl-D-1-methyoxcarbonyltryptophanyl-D-norleucine) (Calbiochem, La Jolla, CA), cremophor EL (Nacalai Tesque, Kyoto, Japan), and KB-R7943 (2-[2-[4-(4-Nitrobenzyloxy)phenyl]ethyl]isothiourea) (Tocris, Ballwin, MO). Fura-2/AM, PDB, ryanodine, Bay K8644, bisindolylmaleimide I, BQ-123, and KB-R7943 were dissolved in dimethyl sulfoxide. ET-1 and sarafotoxin S6c were dissolved in dilute acetic acid (0.1%). The final concentration of the solvent was less than 0.1%, which alone had no effect on $[Ca^{2+}]_i$ or contractile force (data not shown).

2.4. Statistics

The numerical data were expressed as the means ± standstandard error. Differences between mean values were evaluated by unpaired Student's *t*-test. One-way analysis of variance (ANOVA) followed by either Dunnett's test or the Tukey test was adopted for comparisons among more than two groups. A probability of less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of ET-1 on $[Ca^{2+}]_i$ transients and contractile force

ET-1 (1–300 nM) increased the contractile force of neonatal (days 1–2) mouse cardiac muscle in a concentration-dependent manner with a threshold concentration of 1 nM (Fig. 1B) and an EC_{50} value of 3.8×10^{-8} M. The positive inotropy evoked by each concentration of ET-1 reached a plateau within 20–40 min. A representative recording of the experiment is shown in Fig. 1A. Pretreatment of the tissue with a selective endothelin ET_A receptor antagonist, BQ-123 (1 and 10 μM), significantly shifted the concentration–response curve to the right (EC_{50} values: 1 μM BQ-123, 1.3×10^{-7} M; 10 μM BQ-123, 3.7×10^{-7}, Fig. 1B), without any effect on the basal contractile force (to 94.8 ± 1.6% and 98.6 ± 1.1%, respectively, $n = 5$ each). In contrast, a selective endothelin ET_B receptor agonist, sarafotoxin S6c (100 nM), did not affect the contractile force (to 97.7 ± 8.2%, $n = 4$). Furthermore, BQ-788 (1 μM), a selective antagonist of the endothelin ET_B receptor, had no effect on the ET-1-induced positive inotropy.

The pacing frequency affected the contractile amplitude of the prepared neonatal mouse ventricles. Stimulation of the tissues at 2 Hz evoked a greater contraction than that at 5 Hz (100% at 0.5 Hs vs. 190.8 ± 27.3% at 2 Hz, $n = 4$). ET-1 (100 nM) also significantly increased the contractile force to 130.0 ± 8.8% ($n = 4$) at 2 Hz, although the positive inotropic effect of ET-1 was relatively weaker than that at 0.5 Hz. Because ET-1, 300 nM, induced arrythmogenetic effects in three of five preparations, while 100 nM ET-1 induced arrythmogenetic effects in only one of five preparations, the latter concentration was used in subsequent experiments.

To further examine the positive inotropic effect of ET-1, ET-1 (100 nM) was applied to preparations loaded with fura-2. Fig. 2A shows typical recordings of the $[Ca^{2+}]_i$ transients as indicated by the ratio of the fluorescence elicited by 340 and 380 nm, and of the contraction. ET-1 (100 nM) significantly increased the peak $[Ca^{2+}]_i$ transients and the peak contractile force to 163.0 ± 7.6% and 239.5 ± 22.7% of the control level ($n = 6$), respectively (Fig. 2B). The increase in the contractile force was of the same order as that in the absence of fura-2 (250.3 ± 25.9%, $n = 5$). Each value reached a plateau in about 40 min. The averaged tracings of the $[Ca^{2+}]_i$ transients and contraction are shown in Fig. 2C, and the contractile parameters of the effect of ET-1 are summarized in Table 1. The maximum rates of rise of force (dF/dt max) and $[Ca^{2+}]_i$ transients (dR/

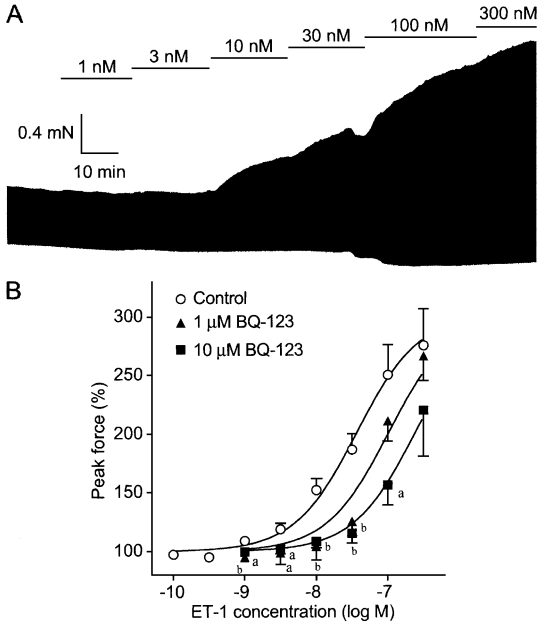

Fig. 1. (A) Representative recording of the change in contractile force induced by the cumulative administration of ET-1. (B) Concentration–response curves for the positive inotropic effects of ET-1 in the neonatal mouse right ventricle in the absence ($n = 5$) or presence of 1 μM ($n = 5$) and 10 μM ($n = 5$) BQ-123. ET-1 (0.1–300 nM) was administered cumulatively as shown in (A). The value obtained just before addition of ET-1 was taken as 100%. [a] and [b]: significantly different from values obtained in the absence of BQ-123 ($P < 0.05$ and $P < 0.01$, respectively).

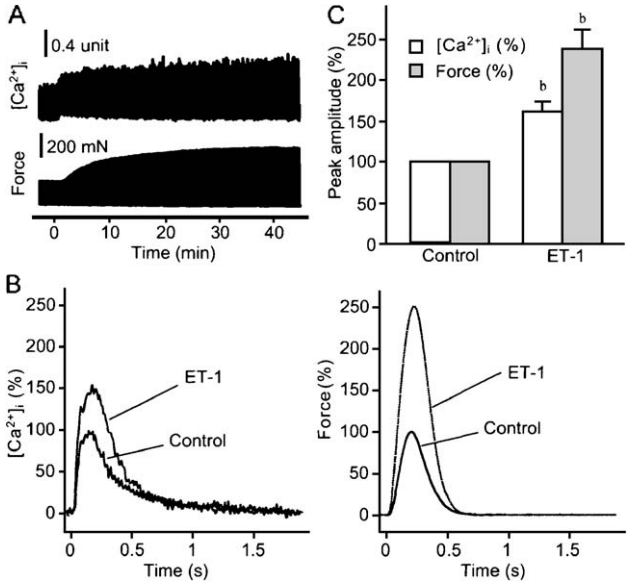

Fig. 2. Effects of ET-1 (100 nM) on $[Ca^{2+}]_i$ transients and the contractile force of the neonatal mouse myocardium loaded with fura-2. (A) Representative tracings of changes in $[Ca^{2+}]_i$ transients and the contractile force elicited by ET-1 in the neonatal mouse right ventricle. The time when 100 nM ET-1 was added is shown as 0 s. (B) Averaged tracings of 30 successive $[Ca^{2+}]_i$ transients (left) and isometric contractions (right) in the absence or presence of 100 nM ET-1. Electrical stimulation was applied at time zero. (C) Effects of ET-1 (100 nM) on the peak values. Each column indicates the mean of the peak amplitude of the $[Ca^{2+}]_i$ transient or the peak contractile force ($n = 6$). Values obtained in the absence of endothelin-1 were taken as 100%. [b]: significantly different from controls ($P < 0.01$).

Table 1
Effect of endothelin-1 (100 nM) on contractile parameters

	Control	Endothelin-1
Peak force (%)	100	239.5 ± 23.2**
d*F*/d*t* max (%)	100	205.0 ± 14.0**
Time to peak force (ms)	174.2 ± 11.8	190.9 ± 12.9
Time to half relaxation (ms)	150.0 ± 25.3	161.4 ± 21.7
Peak $[Ca^{2+}]_i$ transient (%)	100	163.0 ± 7.6**
d*R*/d*t* max (%)	100	140.0 ± 9.5**
Time to peak $[Ca^{2+}]_i$ (ms)	97.0 ± 11.1	130.3 ± 16.2*
Time to half decay (ms)	200.0 ± 15.7	184.9 ± 23.0

* $P < 0.05$ as compared with controls ($n = 6$ each).
** $P < 0.01$ as compared with controls ($n = 6$ each).

d*t* max) and the time to peak $[Ca^{2+}]_i$ (TTPR) were significantly increased by ET-1.

3.2. Correlation between [Ca^{2+}] transients and contractile force

The change in extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) from 2 to 4–8 mM increased the $[Ca^{2+}]_i$ transients and contractile force in a concentration-dependent manner (Fig. 3A), with a concomitant increase in the diastolic levels of $[Ca^{2+}]_i$ (data not shown). The effects of 10 nM and 100 nM ET-1 on the $[Ca^{2+}]_i$ transient and contractile force are superimposed in Fig. 3A; it can be seen that ET-1 did not change the $[Ca^{2+}]_i$–force relationship.

To further clarify the effect of ET-1 on myofilament $[Ca^{2+}]_i$ responsiveness, we compared the trajectories of the $[Ca^{2+}]_i$–force relationship in the presence ($[Ca^{2+}]_o = 2$ mM) or absence ($[Ca^{2+}]_o = 6$ mM) of 100 nM ET-1. In the decay phase of the representative trajectories, the two curves overlapped (Fig. 3B).

3.3. Effects of ryanodine on positive inotropic effects of ET-1

Ryanodine is known to open-lock the ryanodine receptor and is thereby thought to drain the sarcoplasmic reticulum Ca^{2+} store. Ryanodine (1 μM) decreased the contraction 74.1 ± 6.8%, (from 0.32 ± 0.04 to 0.24 ± 0.04 mN) in accordance with the decrease in the peak $[Ca^{2+}]_i$ transient to 74.2 ± 4.1% ($n = 6$, Fig. 4A). The time to peak force (TTPF) was gradually increased from 154.5 ± 3.7 ms (before ryanodine addition) to 209.1 ± 16.2 ms (90 min after the addition). Fig. 4B show representative traces of the effects of 1 μM ryanodine on the $[Ca^{2+}]_i$ transients and contractile force.

ET-1 (100 nM) was applied to the muscle 30 min after the pretreatment with 1 μM ryanodine. Although TTPF and TTPR were not fully increased at 30 min, we added ET-1 at this point because the effect on the peak values was maximal, and because longer pretreatment was considered inappropriate due to the potential leak of fura-2 out of cardiomyocytes. ET-1 increased the peak contractile force and the peak $[Ca^{2+}]_i$ transients to 161.5 ± 18.5% and 252.3 ± 30.0%, respectively ($n = 6$, Fig. 4C). The representative traces for the $[Ca^{2+}]_i$ transients and contractile force 30 min after the application of ET-1 are shown in Fig. 4B. Table 2 shows the summarized results of the effect of ET-1 on contractile parameters. TTPR was longer in the presence than in the absence of ET-1, which may indicate that the 30-min pretreatment period was not long enough for TTPR to reach a plateau.

3.4. Effects of KB-R7943 on the positive inotropic effects of ET-1

We examined the effect of KB-R7943, an inhibitor of the Ca^{2+} influx through the Na^+–Ca^{2+} exchanger, on the positive inotropy induced by reduction of $[Na^+]_o$, which is considered to increase the contractile force by the increase in the Ca^{2+} influx through the Na^+–Ca^{2+} exchanger (Même

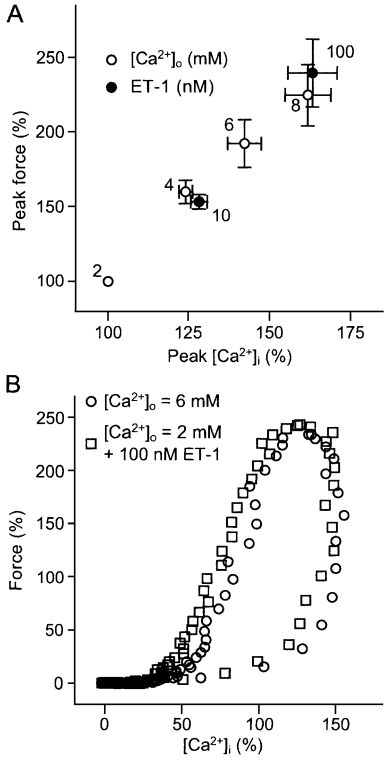

Fig. 3. (A) Relationship between peak amplitude of $[Ca^{2+}]_i$ transient and peak force obtained in the neonatal mouse right ventricle by changing external Ca^{2+} concentration or addition of endothelin-1 (10 nM and 100 nM). The external Ca^{2+} concentration ($[Ca^{2+}]_o$) was changed from 2 to 4, 6, or 8 mM ($n = 5$ each). The effects of ET-1 were observed in the 2 mM $[Ca^{2+}]_o$ PSS. The peak force and peak amplitude of $[Ca^{2+}]_i$ transient obtained in normal PSS ($[Ca^{2+}]_o = 2$ mM) were taken as 100%. Numbers represent $[Ca^{2+}]_o$ or ET-1 concentration. (B) The phase–plane diagram converted from the time-dependent changes in averaged signals of isometric force and fura-2 fluorescence ratio of right ventricular preparations in the absence (open symbols, $[Ca^{2+}]_o = 6$ mM) or the presence of 100 nM ET-1 (closed symbols, $[Ca^{2+}]_o = 2$ mM). Values obtained in normal PSS ($[Ca^{2+}]_o = 2$ mM) were taken as 100%.

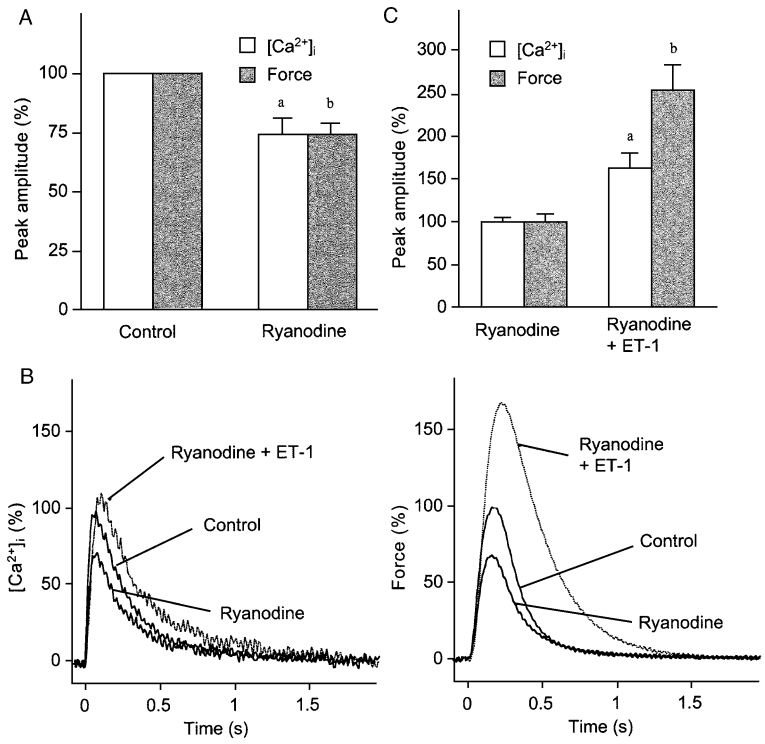

Fig. 4. (A) Effects of ryanodine (1 μM) on the peak values of $[Ca^{2+}]_i$ transients and contractile force of the neonatal mouse myocardium loaded with fura-2 ($n=6$). Values obtained without any treatment were taken as 100%. [a] and [b]: significantly different from controls ($P<0.05$ and $P<0.01$, respectively). (B) Averaged tracing of 30 successive $[Ca^{2+}]_i$ transients (left) and isometric contractions (right) of preparations without any treatment, of preparations treated with ryanodine (1 μM), and of preparations treated with ET-1 (100 nM) additionally in the neonatal mouse right ventricles loaded with fura-2. Stimulation was applied at time zero. (C) Effects of ET-1 (100 nM) on the ryanodine-pretreated preparations. ET-1 (100 nM) was added after 30 min pretreatment of 1 μM ryanodine ($n=6$). Values obtained just before the addition of endothelin-1 were taken as 100%. [a] and [b]: significantly different from controls ($P<0.05$ and $P<0.01$, respectively).

et al., 2001). Fig. 5A shows the positive inotropic effect of low $[Na^+]_o$ stimulations (130 mM and 115 mM) in the absence or presence of KB-R7943 (10 and 30 μM). Reduction of $[Na^+]_o$ from 145 to 130 or 115 mM gradually increased the contractile force until it reached a plateau after 20 min (230.4 ± 23.1% and 414.8 ± 92.1% for 130 mM and 115 mM, respectively, $n=4$ each). KB-R7943 (10 μM) did not affect the positive inotropy induced by low $[Na^+]_o$ stimulation. In the presence of 30 μM KB-R7943, however, the positive inotropy induced by reduction of $[Na^+]_o$ to 130 or 115 mM was significantly inhibited (to 117.9 ± 9.6% and 121.1 ± 4.7%, respectively, $n=6$). In contrast, 30 μM KB-R7943 did not change the basal twitch contractile force (122.9 ± 15.1%, Fig. 5A). Thus, we confirmed that 30 μM KB-R7943 selectively inhibits the contractile force augmented by Ca^{2+} influx through the Na^+–Ca^{2+} exchanger. As shown in Fig. 5B, pretreatment of the muscle with 30 μM KB-R7943 significantly inhibited the positive inotropic effect of 100 nM ET-1 ($n=6$), while 10 μM KB-R7943 did not have a significant effect ($n=6$). Since it is uncertain whether the effects of KB-R7943 are specific to the Na^+–Ca^{2+} exchanger (Reuter et al., 2002), we examined the effects of KB-R7943 on the positive inotropic effect caused by activation of L-type voltage-dependent Ca^{2+} channels. Bay K8644 (10 nM), a potent activator of L-type voltage-dependent Ca^{2+} channels, increased the contractile force to 191.6 ± 7.1% ($n=5$). However, pretreatment of the tissue with KB-R7943 (30 μM) did not affect this positive inotropic effect (to 169.8 ± 10.8%, $n=5$). In this study, we attempted to examine the effect of KB-R7943 on the change in $[Ca^{2+}]_i$. However, we were not

Table 2
Effect of endothelin-1 (100 nM) on contractile parameters in ryanodine (1 μM)-pretreated tissues

	Control: pretreatment with ryanodine for 30 min	Plus endothelin-1 (100 nM)
Peak force (%)	100	252.3 ± 29.7**
d*F*/d*t* max (%)	100	218.1 ± 34.0*
Time to peak force (ms)	166.2 ± 11.7	202.6 ± 16.7**
Time to half relaxation (ms)	110.3 ± 27.3	138.3 ± 48.0
Peak $[Ca^{2+}]_i$ transient (%)	100	161.5 ± 18.5*
d*R*/d*t* max (%)	100	128.6 ± 16.0
Time to peak $[Ca^{2+}]_i$ (ms)	109.9 ± 4.4	122.1 ± 9.1
Time to half decay (ms)	173.4 ± 6.8	165.8 ± 21.9

Muscle strips were treated with ryanodine (1 μM) for 30 min before addition of ET-1 (100 nM).

* $P<0.05$ as compared with controls ($n=6$ each).

** $P<0.01$ as compared with controls ($n=6$ each).

able to measure this effect, because KB-R7943 (30 μM) interfered with the fluorescence parameters of fura-2 (data not shown).

3.5. Effects of PKC inhibitors on the positive inotropic effects of ET-1

Fig. 6A shows the effect of 100 nM PDB, a PKC activator, on contractile force in the mouse neonatal myocardium. PDB significantly decreased the contractile force to 62.3 ± 4.9% (n = 4). This negative inotropic effect of PDB was completely inhibited by 3 μM bisindolylmaleimide I, a PKC inhibitor (Fig. 6A). On the other hand, 10 μM bisindolylmaleimide I alone increased the contractions to 175.3 ± 10.6% (from 0.41 ± 0.07 to 0.70 ± 0.11 mN) in normal PSS (n = 4), indicating that protein kinase C is constitutively activated to suppress the contractions. As demonstrated in Fig. 6B, pretreatment of the tissue with 3 μM bisindolylmaleimide I did not alter the ET-1-induced positive inotropy. The effects of other PKC inhibitors on the ET-1-induced positive inotropy were also examined. Gö6976 is a specific inhibitor of PKCα and PKCβ. Gö6976 (1 μM) suppressed the negative inotropic effect of PDB (100 nM) but did not affect the ET-1 (100 nM)-induced positive inotropy. Rottlerin, on the other hand, is a specific inhibitor of PKCδ and did not have any effect on the actions of PDB (100 nM) and ET-1 (100 nM).

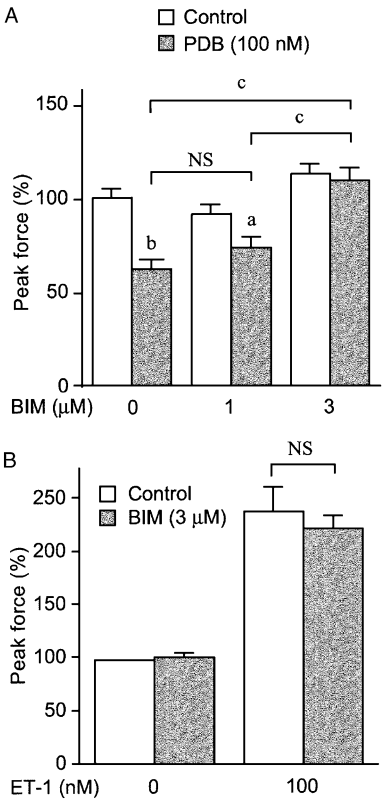

Fig. 6. (A) Effects of PDB (100 nM) on the bisindolylmaleimide I (0–10 μM)-pretreated preparations and (B) effects of the pretreatment with bisindolylmaleimide I (3 μM) on the positive inotropic effect of ET-1 (100 nM). Bisindolylmaleimide I was applied 60 min before the addition of PDB or ET-1. Values obtained in normal PSS are taken as 100%. [a] and [b]: significantly different from controls (P < 0.05 and P < 0.01, respectively). [c]: significant difference between the values (P < 0.01).

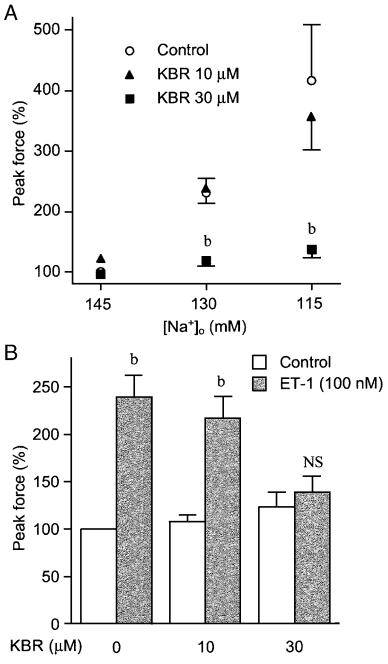

Fig. 5. Effects of KB-R7943 (KBR) on the positive inotropic effects of (A) reduction of $[Na^+]_o$ (n = 4 for control, n = 6 for 10 μM KBR, and n = 6 for 30 μM KBR) and (B) ET-1 (n = 6, each). Tissues were pretreated with KB-R7943 for 45 min before the stimulations with low $[Na^+]_o$ PSS or endothelin-1. Values obtained without any treatments were taken as 100%. [b]: significantly different from controls (P < 0.01). NS: not significantly different from controls.

4. Discussion

In the present study, we demonstrated that ET-1 elicited a positive inotropic effect in the neonatal mouse right ventricle. A selective endothelin ET_A receptor antagonist, BQ-123, shifted the concentration–response curve to the right, but a selective endothelin ET_B receptor antagonist, BQ-788, did not. Furthermore, the selective ET_B receptor agonist, sarafotoxin S6c, did not affect the twitch contractile force. These results suggest that the positive inotropic effect of ET-1 is mediated through the endothelin ET_A receptor in the neonatal mouse right ventricle, as previously shown by Sekine et al. (1999). However, the possible involvement of additional receptor subtypes other than ET_A or ET_B (such as a possibility of post-translational modification of ET_A or ET_B) cannot be excluded (Russell and Molenaar, 2000).

The sensitization of contractile elements to Ca^{2+} is one of the mechanisms of the positive inotropic effect of ET-1

(Krämer et al., 1991). To clarify whether endothelin-1 changes the Ca^{2+} sensitivity of myofilaments in the neonatal mouse right ventricle, we compared the effects of ET-1 and changes in extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) on the relationship between $[Ca^{2+}]_i$ transients and contractile force. The results indicated that ET-1 did not change the $[Ca^{2+}]_i$–force relationship. This conclusion is also supported by the fact that the trajectory of the $[Ca^{2+}]_i$–force relationship in the presence of ET-1 was well matched with that in the absence of ET-1.

In this study, we found that the positive inotropic effect of ET-1 was associated with an increase in the amplitude of the $[Ca^{2+}]_i$ transient. There are at least three elements that we expected to contribute to the increase in $[Ca^{2+}]_i$ in cardiac muscle: (1) an increase in Ca^{2+} release from the sarcoplasmic reticulum; (2) an increase in Ca^{2+} influx through Na^+–Ca^{2+} exchangers; and (3) an increase in Ca^{2+} influx through voltage-dependent Ca^{2+} channels. We first examined the effect of ryanodine on the positive inotropic effect of ET-1 in order to eliminate the effect of Ca^{2+} release from the sarcoplasmic reticulum. In the control neonatal ventricle, the peak $[Ca^{2+}]_i$ transient and contractile force induced by field stimulation was decreased by ryanodine (1 μM) with the increase in TTPF and TTPR, suggesting that ryanodine inhibits the storage of Ca^{2+} in the sarcoplasmic reticulum due to the opening of ryanodine receptor channels. However, ET-1 greatly increased the peak $[Ca^{2+}]_i$ transient and contractile force in the presence of ryanodine, suggesting that the ryanodine-sensitive Ca^{2+} store may not play an important role in the positive inotropic effect of ET-1.

Secondly, we examined the effect of KB-R7943, an inhibitor of the reverse mode (Ca^{2+} influx mode) of Na^+–Ca^{2+} exchangers (Watano et al., 1996; Iwamoto et al., 1996; Yang et al., 1999). Pretreatment of the tissue with 30 μM KB-R7943 significantly inhibited the positive inotropy induced by ET-1 without significant effects on the amplitude of the twitch contractile force in normal PSS, suggesting that the Ca^{2+} influx through Na^+–Ca^{2+} exchanger may play a pivotal role in the positive inotropic effects of ET-1 in mouse neonatal cardiac muscle. Data on the effects of ryanodine and KB-R7943 suggest that the increase in Ca^{2+} influx produced through the Na^+–Ca^{2+} exchanger by ET-1 can induce positive inotropy without the Ca^{2+}-loading function of the sarcoplasmic reticulum in neonatal cardiomyocytes. For example, Haddock et al. (1999) demonstrated that the influx mode of the Na^+–Ca^{2+} exchanger was more important that the sarcoplasmic reticulum for induction of the subcellular $[Ca^{2+}]_i$ transient in neonatal cardiomyocytes of rabbits. Although some researchers (Kimura et al., 1999; Woo and Morad, 2001) have questioned the specificity of KB-R7943 for the calcium influx mode of the Na^+–Ca^{2+} exchanger, the time course of twitch contractions was not affected by KB-R7943 (30 μM) in the present study. This finding suggested that the effects of KB-R7943 on the Ca^{2+} efflux mode of the Na^+–Ca^{2+} exchanger are probably weak in the myocardium of the neonatal mouse.

Although KB-R7943 almost completely inhibited the positive inotropic effect of ET-1, we still cannot rule out the possibility that voltage-dependent Ca^{2+} channels are involved with the effect of ET-1. In fact, there are some reports suggesting that endothelin-1 activates dihydropyridine-sensitive voltage-dependent Ca^{2+} channels (Lauer et al., 1992; Woo and Lee, 1999; He et al., 2000). Furthermore, an outward Kv channel is also a candidate for leading the ET-1-induced positive inotropic effect by prolonging the action potential duration and increasing Ca^{2+} influx, as has been observed in myocytes isolated from cardiac hypertrophy or failure hearts (Xu et al., 2001; Kääb et al., 1996). Further studies are needed to clarify these points.

Since PKC has been reported to be important in the positive inotropic effect of endothelin-1 in many cardiac tissues (Krämer et al., 1991), we also examined whether this enzyme is involved in the mechanism of the effect of ET-1 in neonatal mouse right ventricles. The addition of 100 nM PDB decreased the contractile force, while the addition of 10 μM bisindolylmaleimide I, on the other hand, increased the contraction. Furthermore, pretreatment with 3 μM bisindolylmaleimide I completely inhibited the negative inotropic effect of 100 nM PDB but did not affect the positive inotropy induced by ET-1 (100 nM). These results suggest that the PKC is not involved in the positive inotropic effect of ET-1 in neonatal mouse right ventricles. Though we cannot clarify the involvement of the Na^+–H^+ exchanger in the ET-1-induced positive inotropy, activation of the inward Ca^{2+} current through the Na^+–Ca^{2+} exchanger during an action potential may be induced by a pathway different from those seen in other species, in which the Na^+–H^+ exchanger is activated by PKC.

We have previously reported that the adult mouse (8–10 weeks) right ventricle showed a decrease in contractile force in response to ET-1 concomitant with a decrease in $[Ca^{2+}]_i$ transient (Izumi et al., 2000), suggesting the presence of developmental conversion of inotropic effects in mouse ventricles. Although PKC has been suggested to mediate the negative inotropic effect in adult mice (Izumi et al., 2000), we found that, in the neonatal mouse, PKC was not involved in the mechanism of the positive inotropic effect of ET-1. Because we observed negative inotropic effects on PKC activation in neonatal mouse (Fig. 6A), we speculated that the mechanism responsible for the PKC-induced negative inotropy is already matured in the neonatal mouse heart, but that the link between the endothelin ET_A receptor and PKC may still be immature in neonates.

In conclusion, ET-1 elicited positive inotropy in the neonatal mouse right ventricle via activation of endothelin ET_A receptors. The positive inotropic effect may be mediated by an increase in $[Ca^{2+}]_i$ transient through the activation of Ca^{2+} influx via Na^+–Ca^{2+} exchangers without change in Ca^{2+} responsiveness of myofilaments or Ca^{2+} efflux from the sarcoplasmic reticulum. Furthermore, the

positive inotropic effect of ET-1 in neonatal mouse ventricle is independent of PKC.

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