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Heterogeneity and underlying mechanism for inotropic action of endothelin-1 in rat ventricular myocytes

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1. To clarify the mechanisms underlying the positive inotropic action of endothelin-1 (ET-1), we investigated the effect of ET-1 on twitch cell shortening and the Ca^{2+} transient in rat isolated ventricular myocytes loaded with a fluorescent Ca^{2+} indicator indo-1.

2 There was a cell-to-cell heterogeneity in response to ET-1. ET-1 (100 nM) increased twitch cell shortening in only 6 of 14 cells (44 %) and the increase in twitch cell shortening was always accompanied by an increase in the amplitude of the Ca^{2+} transient.

3 The ET_A- and ET_B-receptors antagonist TAK-044 (100 nM) almost reversed both the ET-1-induced increases in twitch cell shortening and in the Ca^{2+} transient. In the ET-1 non-responding cells, the amplitude of the Ca^{2+} transient never increased.

4 Intracellular pH slightly increased ($\sim$0.08 unit) after 30 min perfusion of ET-1 in rat ventricular myocytes. However, ET-1 did not change the myofilament responsiveness to Ca^{2+}, which was assessed by (1) the relationship between the Ca^{2+} transient amplitude and twitch cell shortening, and by (2) the Ca^{2+} transient-cell shortening phase plane diagram during negative staircase.

5 We concluded that there was a cell-to-cell heterogeneity in the positive inotropic effect of ET-1, and that the ET-receptor-mediated positive inotropic effect was mainly due to an increase in the Ca^{2+} transient amplitude rather than to an increase in myofilament responsiveness to Ca^{2+}.

Keywords: Endothelin-1; TAK-044; myofilament responsiveness; common trajectory: indo-1; ventricular myocyte: Ca^{2+} transient: inotropic action; pH_i

Introduction

Endothelin is a potent vasoactive polypeptide of 21 amino acids and was originally isolated from the medium bathing primary culture of porcine aortic endothelial cells (Yanagisawa *et al*., 1988). Endothelin includes at least three analogues, named endothelin-1 (ET-1), ET-2, and ET-3, and among these families ET-1 has been understood to be the most potent active form (Masaki *et al*., 1991). Within the cardiovascular systems, ET-1 stimulates the secretion of atrial natriuretic peptide (Thibault *et al*., 1994), and causes cardiac hypertrophy (Ito *et al*., 1994) and coronary vasoconstriction (Khandoudi *et al*., 1994). ET-1 has been also shown to produce the positive chronotropic (Ishikawa *et al*., 1988) and inotropic effects in isolated hearts (Khandoudi *et al*., 1994), in isolated cardiac muscle preparations (Wang *et al*., 1991; Takanashi & Endoh, 1992) and in isolated myocytes (Vigne *et al*., 1989; Kelly *et al*., 1990; Krämer *et al*., 1991; Qiu *et al*., 1992; Kohmoto *et al*., 1993; Mebazaa *et al*., 1993). Cultured endocardial endothelial cells of sheep were found to express endothelin-1 mRNA and to release ET-1 into the superfusing solution resulting in an increase in contraction (Mebazaa *et al*., 1993). Endothelin released from vascular or endocardial endothelial cells of rat ventricular trabeculae increases cardiac contractility in the absence of added synthesized peptide (McClellan *et al*., 1995).

It has been proposed that endothelin may play an important role in the pathogenesis of myocardial infarction (Watanabe *et al*., 1991). Abnormal Ca^{2+} handling and the Ca^{2+} overload are thought to play a key role in ischaemia/reperfusion injury (Hayashi *et al*., 1992). In this regard, it is of great interest to investigate the relationship between the positive inotropic action of ET-1 and the modulation of Ca^{2+} in order to understand the role of ET-1 under many pathophysiological conditions.

However, the positive inotropic effect of ET-1 has not been found consistently and a negative chronotropic action of ET-1 has also been observed. (Borges *et al*., 1989; Volkmann *et al*., 1990; Ono *et al*., 1994). These variable responses could be age- and species-related, but it is also likely that the experimental conditions may make a significant difference, e.g. multicellular tissue or isolated myocytes, and the basic inotropic state.

The intracellular mechanisms involved in the positive inotropic effect of ET-1 also remain undefined. Endothelin has been shown to activate phospholipase C (PLC) which increases phosphoinositide hydrolysis and produces two biologically important substances, 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (Hilal-Dandan *et al*., 1992; Van Heugten *et al*., 1994). Protein kinase C (PKC), which is activated by DAG, can stimulate the Na^+/H^+ exchanger, leading to an intracellular alkalization. Intracellular alkalization has been shown to increase myofilament responsiveness to Ca^{2+} (Fabiato & Fabiato, 1978), which is proposed to account for the positive inotropic effect of ET-1. This proposal was based on the observation that ET-1-induced elevation of intracellular pH (pH_i) and the positive inotropic effect of ET-1 were not accompanied by an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in ventricular myocytes (Kelly *et al*., 1990; Qiu *et al*., 1992; Kohmoto *et al*., 1993).

A close relationship between ET-1-induced phosphoinositide hydrolysis and the positive inotropic effect of ET-1 was demonstrated in rabbit ventricular myocardium (Takanashi & Endoh, 1992). Although the role of IP_3 in the intracellular

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signalling systems in myocardium is not clearly understood, an IP_3-mediated increase in $[Ca^{2+}]_i$ could be involved in the positive inotropic effect of ET-1. Mebazaa *et al.* (1993) showed that the positive inotropic effect of ET-1 was associated with an increased Ca^{2+} transient amplitude. In other studies, ET-1 increased both $[Ca^{2+}]_i$ and the myofilament responsiveness to Ca^{2+} (Wang *et al.*, 1991; Fujita & Endoh, 1996). Therefore, it is still unclear whether the change in myofilament responsiveness to Ca^{2+} or the change in Ca^{2+} metabolism is the mechanism for the inotropic action of ET-1.

In this study, we investigated the heterogeneous responses to ET-1 in rat isolated myocytes and then studied the mechanisms underlying the positive inotropic effect of ET-1. The changes in $[Ca^{2+}]_i$ were monitored simultaneously with twitch cell shortenings in isolated myocytes. We demonstrate that (1) there was a cell-to-cell heterogeneity in the positive inotropic effect of ET-1, (2) the small increase in pH_i induced by ET-1 would not be enough to change the myofilament responsiveness to Ca^{2+}, and (3) the increase in the amplitude of the Ca^{2+} transient may be the main mechanism for the positive inotropic effect of ET-1.

Methods

Isolation of ventricular myocytes

Ventricular myocytes were isolated from male rats (200–240 g) by the method described previously (Hayashi *et al.*, 1992). Briefly, the heart was quickly excised, attached to the bottom of a Langendorff column and perfused with the solutions gassed with 95% O_2 and 5% CO_2 and maintained at 34 ± 0.5°C (pH 7.4). The first perfusate was a Ca^{2+}-free Krebs solution of the following composition (in mM): NaCl 113.1, KCl 4.6, $MgCl_2$ 1.2, NaH_2P0_4 3.5, $NaHCO_3$ 21.9 and glucose 10. After 6–8 min of the initial perfusion, 100 ml of Ca^{2+}-free Krebs solution containing enzyme (Collagenase S-l, 300 units ml^{-1}, Nitta, Osaka, Japan) was added to the column and perfused for a further 10–12 min. Finally, the modified Kraftbrühe (KB) solution was introduced to wash out the residual enzyme solution in the heart. Composition of the modified KB solution was as follows (in mM); KOH 70, KCl 40, KH_2P0_4 20, $MgCl_2$ 3, glutamic acid 50, glucose 10, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) 10, ethyleneglycol-bis(b-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid (EGTA) 0.5 (pH 7.4 with KOH) (Yazawa *et al.*, 1990). The ventricles were excised and minced in the modified KB solution with iris scissors and filtered through a mesh to obtain isolated myocytes. When the cells were used, the modified KB solution was changed to HEPES solution containing 1.5 mM $CaC1_2$.

Loading of fluorescent indicators

The myocytes were loaded with 5 μM indo 1-acetoxymethyl ester (indo 1-AM) for 10 min at room temperature. The stock solution of indo 1-AM was prepared by mixing 1 mg of dye in dry dimethyl sulphoxide (DMSO) and was kept frozen in aliquots until use. Immediately before use, dyes were dissolved with KB solution containing 1% bovine serum albumin and 0.075% Pluronic F-127 (wt/vol, final concentration). The myocytes were then washed twice in a modified KB solution and were incubated for an additional 30 min at room temperature for complete hydrolysis of dyes. For the measurement of pH_i, the cells were loaded with 2′,7′-bis (carboxyethyl)-5,6-carboxyfluorescein (BCECF)-AM (0.5 μM) for 30 min and were then washed and incubated as mentioned above.

Apparatus

After fluorescent indicators were loaded, a small portion of myocytes were placed in an experimental chamber mounted on the stage of an inverted microscope (TMD, Nikon, Tokyo, Japan) and perfused with HEPES solution (pH 7.4) at room temperature. The myocytes were stimulated with a bipolar Ag-AgCl electrode by field stimulation at a basic cycle length of 0.5 Hz. The cells were illuminated by a transmitted illuminator or ultraviolet light via an epifluorescence illuminator from a 100-W xenon lamp equipped with an interference filter.

For the simultaneous measurement of $[Ca^{2+}]_i$ and cell length in single myocytes, we designed an optical system with an inverted microscope. To measure $[Ca^{2+}]_i$, indo-1 loaded myocytes were excited at 360 nm. The indo-1 emission signal was separated into 405- and 485-nm wavelengths with the appropriate dichroic mirror and band-pass filters. Both fluorescent signals were sampled by use of two photomultipliers (model R1332, Hamamatsu Photonics, Hamamatsu, Japan) with a photon counting unit (model C3866, Hamamatsu Photonics). The fluorescent ratios were obtained by dividing the fluorescent intensity at 405 nm by the fluorescent intensity at 480 nm after each background subtraction. The cells were simultaneously illuminated with red light (>600 nm) through the normal bright-field illumination optics. The bright-field image was separated from the fluorescence image by 580 nm long-pass dichroic mirror. The cell image was focused on the linear image sensor (model S3904-2048Q, Hamamatsu Photonics) consisted of 2048 photodiodes. Light intensity of each photodiode was scanned at a rate of 1.5 μs/photodiode and monitored on the oscilloscope continuously. Both edges of a cell were identified by the edge detection system (model MOS-SPL 46A001, Hamamatsu Photonics) and cell lengths were computed from the numbers of photodiodes between each edges. Since we used $\times 40$ phase contrast objective lens (Fluor 40 Ph3DL, Nikon) and $\times 1.5$ relay lens and width of each photodiode was 25 μm, the spatial resolution of the system was 0.44 μm. Signals from the photomultipliers and the edge detection system were monitored and stored in a microcomputer (PC-9801 DA, NEC, Tokyo, Japan) at the sampling frequency of 5 ms via a 12 bit AD converter and off-line analysis was conducted.

In the measurement of pH_i, the fluorescent signal was imaged with a silicon-intensified target camera (model C2400, Hamamatsu Photonics), with the output digitized to a resolution of 512×512 pixels by an image analysis system (Argus 50, Hamamatsu Photonics). The exciting wavelengths were 490 and 450 nm and an emission wavelength was at 505–560 nm. Each image was the accumulation of 8 ($30\ s^{-1}$) successive video frames and the fluorescence intensity was measured in a defined cell area. Fluorescence ratios were obtained by dividing, pixel by pixel, the 490 nm image by the 450 nm image after each background subtraction. In order to minimize photobleaching, exposure to excitation light was limited during data collection (0.27 s/each collection) by an electrically controlled shutter, and neutral density (ND) filters were placed in the exciting light paths to prevent photobleaching.

Calibration of BCECF fluorescence

We conducted *in vivo* calibration, in the preliminary group of myocytes, according to the method previously described (Hayashi *et al.*, 1992). BCECF-loaded myocytes were perfused

with the calibration solutions which contained 10 μM nigericin in (mM): KCI 130, $MgCl_2$ 1, 2-(N-morpholino) ethanesulphonic acid 15 and HEPES 15. The pH was adjusted appropriately with KOH. Fluorescence ratios were linearly related to pH_i from 6.5 to 7.5.

Estimation of myofilament responsiveness to Ca^{2+}

The myofilament responsiveness to Ca^{2+} was assessed by (1) the relationship between the amplitude of the Ca^{2+} transients and twitch cell shortening, and by (2) the Ca^{2+} transient-cell shortening phase plane diagram during variable contraction states (Spurgeon *et al*., 1992). In order to vary the amplitudes of the Ca^{2+} transients and to obtain the resulting changes in twitch shortening, we resorted to an usually observed phenomenon in rat myocytes, the post-rest potentiation and the subsequent decay.

Reagents

Cyclo [D-a-asparty-3[(4-phenylpiperazin-1-yl)carbonyl]-L-alanyl-L-a-aspartyl-D-2-(2-thienyl) glycyl-L-leucyl-D-tryptophyl]disodium (TAK-044) was a generous gift from Toshifumi Watanabe Ph.D. of Takeda Chemical Industries, Ltd. (Osaka, Japan). ET-1 was purchased from Peptide Institute Inc. (Osaka, Japan) and was reconstituted in 0.1% acetic acid to make a stock solution (100 μM). Indo-1-AM and BCECF-AM were supplied by Molecular Probes. These reagents were used from stock solutions in ethanol or DMSO. Each reagent produced neither changes in cellular autofluorescence nor fluorescence artifact by itself.

Statistical analyses

Results are expressed as means $\pm$ s.e. for the indicated number (n) of isolated myocytes. Statistical analyses were performed by use of Student's t test, one way analysis of variance (ANOVA), or one way repeated measures ANOVA. Multiple group comparison was carried out by use of Bonferroni-modified t test. The probability was considered significant at $P<0.05$.

Results

Heterogeneity in the positive inotropic effect of ET-1

To investigate the underlying mechanisms for the positive inotropic effect of ET-1, the changes in $[Ca^{2+}]_i$ (the Ca^{2+} transients; estimated with the indo-1 fluorescence ratio) were monitored simultaneously with twitch cell shortenings before and during the perfusion with ET-1. ET-1 (1 and 10 nM; $n=8$ in each concentration) did not cause significant changes in twitch cell shortening and the Ca^{2+} transients. However, 2 cells out of 8 cells which were applied with 10 nM ET-1 showed a slight positive inotropic effect ($\sim 10-15\%$). We used the high concentration (100 nM) of ET-1 (Kelly *et al*., 1990) in the following study, since the purpose of this study was to investigate the underlying mechanism for the inotropic action of ET-1.

Interestingly, only 44% (6 of 14) of myocytes showed an increase in twitch cell shortening while the remaining 8 cells did not respond to ET-1 (100 nM). There was no significant difference in twitch cell shortening or the diastolic and systolic $[Ca^{2+}]_i$ during the control condition between ET-1 responding and non-responding cells (Table 1). Furthermore, twitch cell shortening in the non-responding cells increased after the subsequent exposure to isoprenaline (1 μM; data not shown). Therefore, it is unlikely that the cell-to-cell heterogeneity in the response to ET-1 was due to the difference in the basic characteristics of the myocytes.

Effects of ET-1 on twitch cell shortening and the Ca^{2+} transient

Figure 1A shows a representative example of the effects of ET-1 (100 nM) and TAK-044 (100 nM), an ET_A- and ET_B-receptor antagonist (Watanabe *et al*., 1995), on twitch cell shortening in a rat myocyte. The perfusion of ET-1 increased twitch cell shortening gradually and arrhythmias were recorded after 20 min (indicated in the figure). The subsequent addition of TAK-044 reduced the ET-1-induced increase in twitch cell shortening and eliminated arrhythmias. The washout of TAK-044 in the continuous presence of ET-1 evoked a positive inotropic effect again. TAK-044 alone did not cause any negative inotropic effects in rat ventricular myocytes ($n=5$).

The simultaneous records of the Ca^{2+} transients and twitch cell shortenings in Figure 1B were obtained at the times indicated by the letters in Figure 1A during the control condition (a), 10 min after the perfusion of ET-1 (b), and 15 min after the application of TAK-044 (c), respectively. Figure 1B clearly demonstrates that the positive inotropic effect of ET-1 was always accompanied by an increase in the amplitude of the Ca^{2+} transients. TAK-044 almost completely reversed both the enhancement in the amplitudes of twitch cell shortening and the Ca^{2+} transient.

The averaged augmentation of twitch cell shortening and the Ca^{2+} transient was analysed from 6 pooled cells which responded to ET-1 (Figure 2). ET-1 increased both twitch cell

Table 1 Cell shortening and fluorescent ratio before or after the addition of ET-1

	n	*Control length* (μm)	*Control shortening* (μm)	*CaT base*		*CaT peak*		*CaT amplitude*	
				Before	*After*	*Before*	*After*	*Before*	*After*
Responding cell	6	137.3 $\pm$ 9.5	5.2 $\pm$ 0.7	0.418 $\pm$ 0.045	0.408 $\pm$ 0.051	0.716 $\pm$ 0.053	0.854 $\pm$ 0.071†*	0.298 $\pm$ 0.029	0.433 $\pm$ 0.045†*
Non-responding cell	8	139.9 $\pm$ 8.5	5.9 $\pm$ 0.5	0.407 $\pm$ 0.025	0.404 $\pm$ 0.027	0.723 $\pm$ 0.041	0.714 $\pm$ 0.037	0.316 $\pm$ 0.031	0.309 $\pm$ 0.029

Rat isolated ventricular myocytes loaded with indo-1 were perfused with the control HEPES solution and the solution containing ET-1 (100 nM) with 1 mM $CaC1_2$ and stimulated at 0.5 Hz at room temperature. The Ca^{2+} transients and twitch cell shortenings were recorded simultaneously, and the values of the Ca^{2+} transients were expressed as the ratio of indo-1 fluorescence. Responding cells were defined as the cells which showed the positive inotropic effect after ET-1 was perfused (>10% increase of cell shortening was considered to be the positive inotropic effect). CaT: the Ca^{2+} transient; base: the base of indo-1 ratio during twitch stimuli; peak: the peak of indo-1 ratio; amplitude: the amplitude of indo-1 ratio; after: the values after 10 min perfusion of ET-1. †$P<0.01$ vs before the addition of ET-1 by paired t test *$P<0.01$ vs non-responding cell by non-paired t test.

shortening (Figure 2a) and the Ca^{2+} transient amplitude (Figure 2b) to 234±21% and 146±10% of the control, respectively ($P<0.01$). TAK-044 reversed both the increases in cell shortening (to 124±14%, $P<0.05$ vs ET-1) and in the Ca^{2+} transient amplitude (to 108±17%, $P<0.05$ vs ET-1). In 2 of 6 cells, arrhythmias were observed during the perfusion of ET-1 and those were abolished by the addition of TAK-044. In myocytes where a positive inotropic effect was not induced,

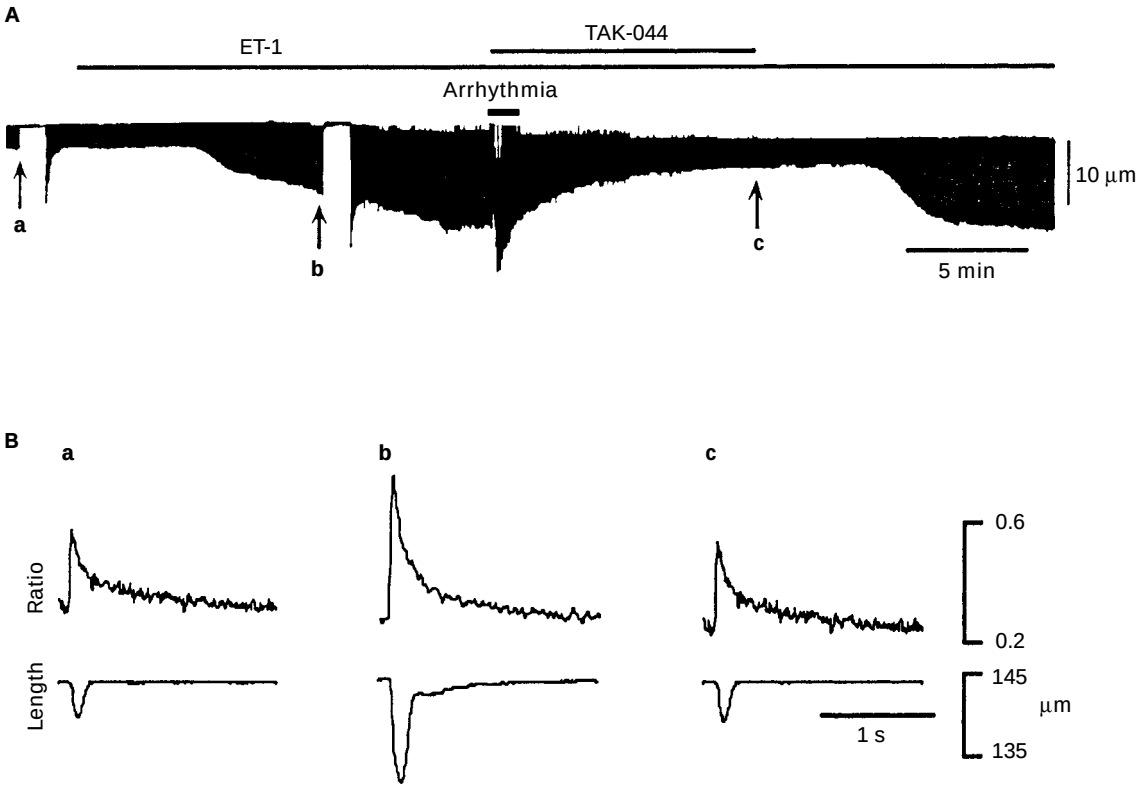

Figure 1 Effects of endothelin-1 (ET-1) and cyclo[D-a-asparty-3[(4-phenylpiperazin-1-yl)carbonyl]-L-alanyl-L-a-aspartyl-D-2-(2-thienyl)glycyl-L-leucyl-D-tryptophyl] disodium (TAK-044) on twitch cell shortening and the Ca^{2+} transients in rat ventricular myocytes loaded with indo-1. Cells were perfused with HEPES solution containing 1 mM $CaCl_2$ and stimulated at 0.5 Hz at room temperature. (A) The tracing shows a continuous record of cell shortening from a typical experiment. ET-1 (100 nM) and TAK-044 (100 nM) were added to the solution as indicated by the bars. Stimuli were stopped for 1 min just after recording (a) and (b) to induce the post rest potentiation in the control solution and in the solution containing ET-1 (see the blank areas in A). (B) Tracings represent the Ca^{2+} transients and simultaneously recorded cell shortening obtained at the times indicated by the letters in (A), during control perfusion (a), 10 min after the addition of ET-1 (b) and 15 min after the addition of TAK-044 in the continuous presence of ET-1 (c).

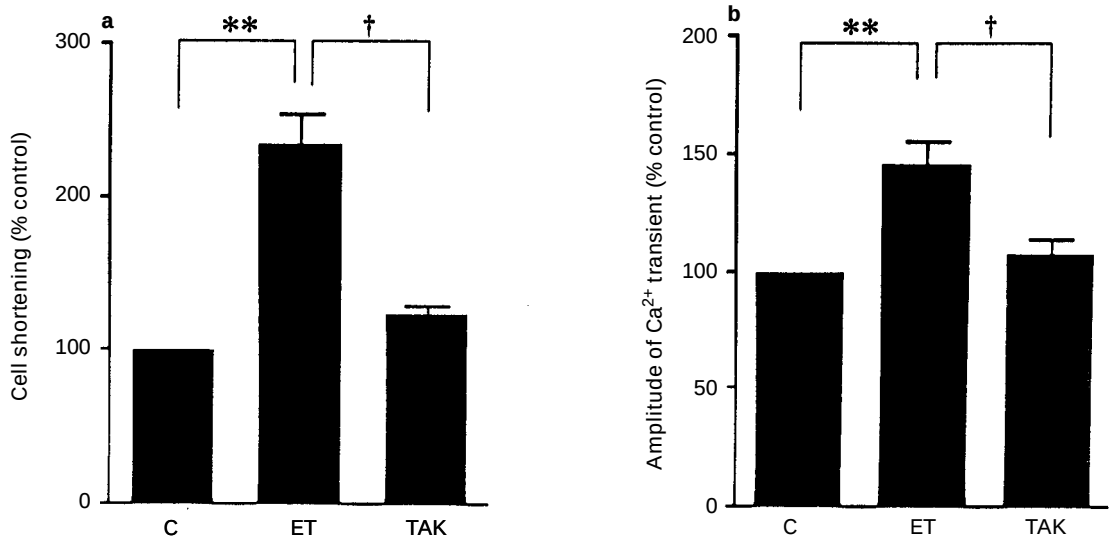

Figure 2 Changes in cell shortening (a) and the amplitude of the Ca^{2+} transients (b) after the exposure to ET-1 and TAK-044 in the presence of ET-1. Cell shortening and the amplitude of the Ca^{2+} transient were expressed as % of the control. C, control; ET, 10 min after the addition of ET-1 (100 nM). TAK, 15 min after the addition of TAK-044 (100 nM) in the continuous presence of ET-1. Values are means±s.e. of 6 myocytes. ** $P<0.01$ vs control, † $P<0.05$ vs ET-1, by ANOVA.

ET-1 never increased the amplitude of the Ca^{2+} transients (see Table 1).

The findings from these experiments are that (1) a heterogeneous response to ET-1 was observed in rat isolated myocytes, (2) the positive inotropic effect of ET-1 was mediated by the ET receptor and (3) the positive inotropic effect of ET-1 always occurred concomitantly with an increase in the amplitude of the Ca^{2+} transients.

Effects of ET-1 on pH_i and myofilament responsiveness to Ca^{2+}

Next, we studied the effect of ET-1 on pH_i in rat ventricular myocytes. There was also heterogeneity in the response to ET-1; 6 cells out of 10 cells showed an increase in pH_i, while 4 cells did not. Figure 3 shows the changes in pH_i in all 10 cells. The pH_i increased significantly after 24 min perfusion of ET-1 (100 nM) and reached 7.34 ± 0.04 ($P < 0.01$) at 30 min.

Finally, since this small but significant increase in pH_i could elevate the myofilament responsiveness to Ca^{2+} and could be a cause for the positive inotropic effect of ET-1, the effect of ET-1 on the myofilament responsiveness to Ca^{2+} was examined in rat isolated myocytes. Figure 4a and b show the simultaneous records of the postrest twitch cell shortenings and the Ca^{2+} transients in the control solution and at 10 min after the addition of ET-1. When a rat myocyte was stimulated following a certain rest period (in our case 60 s), the first postrest twitch cell shortening and the Ca^{2+} transient were potentiated and the following ones decayed toward the steady-state levels. In Figure 4c, the relationships between the amplitude of the Ca^{2+} transients and cell shortening in the control and in the solution containing ET-1 were plotted by use of the data shown in Figure 4a and b, and the relationships were compared by linear regression analysis. It is apparent that ET-1 did not alter the relationship between the amplitudes of the Ca^{2+} transients and twitch cell shortening and there was no significant difference in the regression slopes in the presence or absence of ET-1 (57.9 ± 13.0 in control, 52.5 ± 10.2 in ET-1, $n = 4$, NS).

Recently, it was shown that the relaxation phase of the Ca^{2+} transient-cell shortening phase plane diagram followed a common trajectory independently of the inotropic state of myocytes, and could reflect the relative myofilament responsiveness to Ca^{2+} (Spurgeon *et al*., 1992). As shown in Figure 4d, ET-1 did not shift the common trajectory of the relaxation phase. In 3 of 4 cells, the common trajectory of the relaxation phase was not shifted after the exposure to ET-1 and in one remaining cell, the common trajectory of the relaxation was shifted leftward. Nevertheless, when pH_i was increased by ~ 0.35 unit with 5 min perfusion of the solution containing NH_4Cl (15 mM), the common trajectory was shifted to the left and upward, validating the method employed in this study (data not shown).

These results imply that the small increase in pH_i observed in this study could not explain the positive inotropic effect of ET-1 with the change in the myofilament responsiveness to Ca^{2+}.

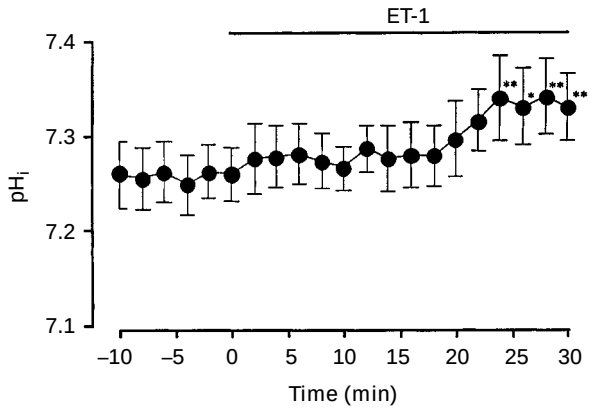

Figure 3 Changes in pH_i in rat ventricular myocytes loaded with BCECF during the perfusion of ET-1 (100 nM). Cells were perfused with HEPES solution containing 1.5 mM $CaCl_2$ and stimulated electrically at 0.5 Hz at room temperature. ET-1 (100 nM) was added to the solution as indicated by the bar. Values are means of 10 myocytes; vertical lines show s.e. $^*P < 0.05$ and $^{**}P < 0.01$ vs control, by one way repeated measures ANOVA.

Discussion

In the present study, we attempted to clarify the mechanisms underlying the positive inotropic effect of ET-1 in rat isolated ventricular myocytes. The main findings of this study are as follows; (1) there was a heterogeneity in response to ET-1 in rat

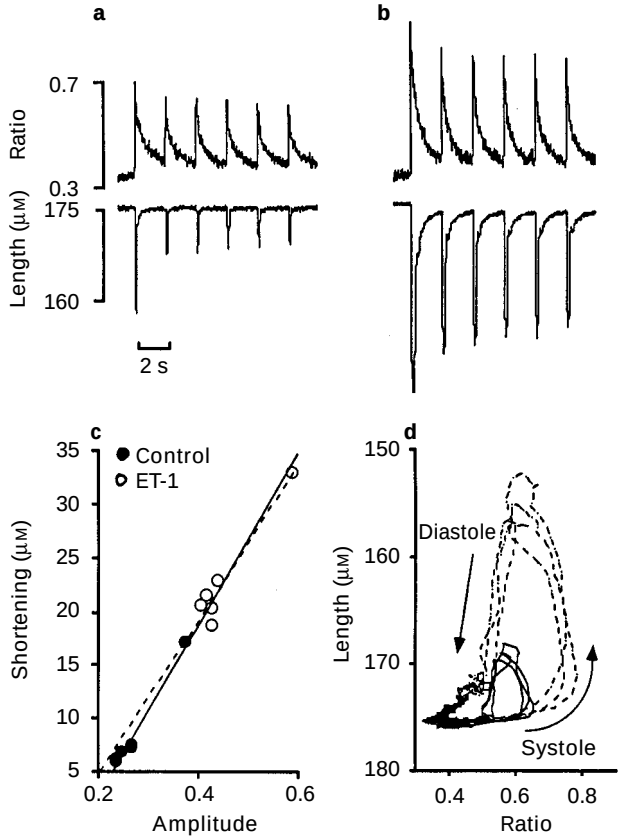

Figure 4 Effect of ET-1 on the myofilament responsiveness to Ca^{2+}. Cells were perfused with a HEPES solution containing 1.5 mM $CaCl_2$ at room temperature. Stimulation at 0.5 Hz was resumed after a certain period of rest (60 s). (a) and (b) Representative records of the postrest potentiation and the subsequent negative staircase of cell shortening and the Ca^{2+} transients (see text), in the control (a) and after 10 min exposure to ET-1 (100 nM; b). (c) The relationship between the Ca^{2+} transient amplitude and cell shortening was obtained from the records of panel (a) and (b). In (c): control was obtained from (a), ET-1 was obtained after the exposure to ET-1 (b). Linear regression analyses were performed and fitted lines were indicated. Solid line; control. Dotted line; after the exposure to ET-1. (d) Phase plane diagram was converted from the data of panel (a) and (b); solid lines; control (from a); dotted lines, after the exposure to ET-1 (b).

ventricular myocytes, (2) the positive inotropic effect of ET-1 was accompanied by an increase in the Ca^{2+} transient amplitudes but was not associated with a change in the myofilament responsiveness to Ca^{2+}.

Our results demonstrated that ET-1 did not evoke the positive inotropic effect constantly in rat ventricular myocytes, and approximately one half of the myocytes responded to the high concentration of ET-1 (100 nM). Although the positive inotropic effect of ET-1 has been described in various types of mammalian myocardium, several studies have indicated no significant inotropic effect of endothelin (Borges *et al*., 1989; Volkmanm *et al*., 1990). Moreover, it has been found that about one half of the cells had limited inotropic action after the exposure of ET-1 in rat ventricular myocytes (Kelly *et al*., 1990) and that ET-1 produced by the endocardial endothelial cell failed to show the positive inotropic effect in 4 of 21 rat ventricular myocytes. (Mebazaa *et al*., 1993).

The present study with rat isolated myocytes revealed that myocytes from the same animal were apparently divided into two groups according to the response to ET-1, without any significant differences in the basic morphological or contractile characteristics under the control condition (Table 1). In the ET-1 non-responding group, the subsequent addition of isoprenaline caused a positive inotropic effect, indicating that these cells reserved a potential to increase contraction in response to another receptor-mediated inotropic stimulation. Therefore, the changes in membrane receptor status or intracellular signalling could contribute to the heterogeneous response to ET-1.

It is clearly shown in this study that in the ET-1 responding cells, the positive inotropic effect of ET-1 was associated with an increase in the amplitude of the Ca^{2+} transients. Since these changes were almost abolished by TAK-044 (Figure 2a,b), the positive inotropic effect was confirmed to be ET receptor-mediated. Our observation that the positive inotropic effect of ET-1 was attributable to an increase in the amplitude of the Ca^{2+} transients is consistent with the previous observations in rat ventricular myocytes (Mebazza *et al*., 1993; Damron *et al*., 1993), atrial myocytes (Vigne *et al*., 1989) and in rat cultured neonatal myocytes (Xu *et al*., 1993).

Several pathways could be responsible for the ET-1-induced increase in the Ca^{2+} transient. The main pathways that Ca^{2+} enters the cytosol during twitch are across the sarcolemma (L-type Ca^{2+} channel or Na^+/Ca^{2+} exchange) and from the sarcoplasmic reticulum (SR). As for L-type Ca^{2+} channel (I_{Ca}), ET-1 increased rabbit ventricular I_{Ca} via a G-protein (Lauer *et al*., 1992). Endothelin has also been found not to inhibit I_{Ca} in guinea pig ventricular myocytes (Habuchi *et al*., 1992), but to inhibit I_{Ca} in guinea pig atria (Ono *et al*., 1994). Furthermore, Kelso *et al*. (1996) have shown the concentration-dependent dual effects (both an increase and decrease of I_{Ca}) of ET-1 in rabbit ventricular myocytes.

Multiple signal transduction pathways were considered to be involved in the actions of ET-1. Hilal-Dandan *et al*. (1992) demonstrated that ET-1 acts via at least two distinct signalling pathways, which are the stimulation of phosphoinositide hydrolysis and the reduction of cyclic AMP accumulation. Therefore, it is possible that the post receptor Ca^{2+} response mediated by ET-1-triggered signal transduction could vary from cell-to-cell, resulting in a heterogeneous response to ET-1. In rabbit ventricular myocytes, phosphoinositide hydrolysis has been considered to be closely linked to the positive inotropic effect of ET-1 (Takanashi & Endoh, 1992). Wang *et al*. (1993) showed that the intracellular IP_3 concentrations were significantly elevated by endothelin stimulation in ferret myocardium. In smooth muscle, it has been established that IP_3 triggers Ca^{2+} release from the intracellular store and contributes to vasoconstriction (Highsmith *et al*., 1992). The role of IP_3 in the intracellular signalling system in myocardium is not clearly understood and it is still controversial whether or not IP_3 can mobilize Ca^{2+} from the SR (Movsesian *et al*., 1985; Nosek *et al*., 1986). In addition to the action of IP_3, another ET receptor-mediated intracellular second messenger, diacylglycerol (DAG) and the subsequent activation of PKC could also alter the intracellular Ca^{2+} handling by modulating the SR function (Movsesian *et al*., 1984).

In our study, ET-1 increased pH_i, presumably from the stimulation of Na^+/H^+ exchanger via the PKC-induced phosphorylation. The increased activity of Na^+/H^+ exchanger could elevate intracellular Na^+ concentration and could lead to a rise in $[Ca^{2+}]_i$ by increasing Ca^{2+} influx via the Na^+/Ca^{2+} exchanger (or reduced Ca^{2+} extrusion via the exchanger). On the other hand, several studies have proposed the contribution of Na^+/H^+ exchanger-induced intracellular alkalosis via the activation of PKC to the positive inotropic effect of ET-1. Intracellular alkalization sensitizes cardiac myofilaments to intracellular Ca^{2+} and increases contractile amplitude and developed tension (Fabiato & Fabiato, 1978). We verified that NH_4Cl-evoked intracellular alkalization could shift the common trajectory to the left and upward. In a study conducted with rat perfused heart, methylisobutyl amiloride (MIA) attenuated the positive inotropic effect of ET-1 (Khandoudi *et al*., 1994). Krämer *et al*. (1991) showed that amiloride prevented the increase in pH_i in response to endothelin and reduced the inotropic response by 45 % in rat myocytes. It has also been shown that MIA decreased but did not eliminate the inotropic action of endothelin in ferret papillary muscles (Wang *et al*., 1993).

In the present study, ET-1 elevated pH_i by ~ 0.08 and this small rise of pH_i became significant after 24 min of the exposure to the peptide (Figure 3). On the other hand, ET-1 showed a positive inotropic effect ($>10\%$ increase of cell shortening was considered to be the positive inotropic effect) about 7.5 min after the perfusion of ET-1. Although we did not measure cell shortening and pH_i simultaneously, it should be noted that there was a certain temporal dissociation between the positive inotropic and the alkalizing effects of ET-1. Furthermore, we demonstrated that ET-1 did not change the myofilament responsiveness to Ca^{2+} (Figure 4). In contrast to our results, Fujita & Endoh (1996) found that in rabbit ventricular myocytes, ET-1 shifted the common trajectory to either upward or up and leftward. They also demonstrated that the slope of peak Ca^{2+} and peak shortening relationship was increased by ET-1.

Although the reasons for these variable results cannot be clearly explained, one possible explanation is the difference in experimental conditions. We used 10^{-7} M ET-1 and observed an increase in the amplitude of the Ca^{2+} transients. Krämer *et al*. (1991) found that in rat myocytes 10^{-9} M ET-1 elevated pH_i with a significant increase in contractility. Touyz *et al*. (1996) showed that ET-1 increased systolic $[Ca^{2+}]_i$ dose-dependently but the concentration of 10^{-9} M was not enough to increase $[Ca^{2+}]_i$ amplitude. Therefore, the difference in the concentration of ET-1 used in these studies could be responsible for the conflicting observations. However, there was no significant positive inotropic effect at the concentrations of 10^{-9} and 10^{-8} M in this study. Furthermore, when a bicarbonate-containing buffer was used, the bicarbonate-related pH_i regulation, such as Cl^-- HCO_3- exchange would diminish the ET-1-induced alkalization. Since we used a HEPES buffered solution (nominally HCO_3^--free) in this study, the small and slow increase in pH_i truly reflects the ET-1-induced activation

of Na^+/H^+ exchange. Krämer *et al*. (1991) demonstrated that the ET-1-induced increases in pH_i and contractile amplitude in a bicarbonate-buffered solution were qualitatively similar to those in a HEPES-buffered solution. Furthermore, several studies have shown that ET-1 in a bicarbonate-buffered medium increased the peak Ca^{2+} transient and contraction (Vigne *et al*., 1989; Touyz *et al*., 1996).

PKC is known to phosphorylate troponin I and troponin T resulting in the modulation of the myofilament responsiveness to Ca^{2+}. Gwathmey & Hajjar (1990) showed that the activation of PKC decreased twitch force in human myocardium associated with a decrease in the responsiveness of the myofilament to Ca^{2+}. On the other hand, Capogrossi *et al*. (1990) showed that the negative inotropic effect of the phorbol ester was due to the diminished amplitude of the Ca^{2+} transients and not due to a decrease in the myofilament responsiveness to Ca^{2+}. It has been postulated that the balance of counter-acting effects induced by PKC, such as the phosphorylation of thin myofilament (decreasing the Ca^{2+} responsiveness) and the activation of Na^+/H^+ exchanger and the subsequent alkalosis (increasing the Ca^{2+} responsiveness), could determine the overall responsiveness to Ca^{2+}. Therefore, the lack of effect of ET-1 on the myofilament responsiveness to Ca^{2+} presented in this study (Figure 4) could be explained if the Ca^{2+} responsiveness was increased due to elevated pH_i, and that was opposed by phosphorylation of thin filament causing the opposite effect and resulting in a net unaltered sensitivity of myofilament to Ca^{2+}. In addition to the DAG-PKC related pathway, IP_3 could also modulate the myofilament responsiveness to Ca^{2+}. In this respect, the effect of IP_3 has been shown to increase or not to increase the myofilament responsiveness to Ca^{2+} (Nosek *et al*., 1986; Wang *et al*., 1993).

Our result in rat cardiomyocytes demonstrated that there was a certain temporal dissociation between the time course of the positive inotropic effect and the changes in pH_i. Taken together with the results that the Ca^{2+} transient amplitude to cell shortening relationship and the common trajectory were not changed after the exposure to ET-1, we concluded that, under our experimental conditions, it is unlikely that the positive inotropic effect of ET-1 was due to the increased myofilament responsiveness to Ca^{2+}.

We concluded that, in our experimental conditions, there was a cell-to-cell heterogeneity in the ET-1-induced positive inotropic action and the positive inotropic effect of ET-1 was due to an increased Ca^{2+} transient amplitude rather than an increased myofilament responsiveness to Ca^{2+}. Further studies are required to clarify the mechanisms responsible for the increase in the Ca^{2+} transient amplitude.

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