British Journal of Pharmacology (2007) 152, 456–463
 0007–1188/07 $30.00
www.brjpharmacol.org

RESEARCH PAPER

Mechanisms of endothelin-1-induced decrease in contractility in adult mouse ventricular myocytes

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Background and purpose: The potent vasoconstrictor polypeptide endothelin-1 (ET-1) plays an important pathophysiological role in progression of cardiovascular diseases and elicits prominent effects on myocardial contractility. Although ET-1 produces a positive inotropy in cardiac muscle of most mammalian species, it induces a sustained negative inotropy in mice. This study was performed to gain an insight into the cellular mechanisms underlying the negative inotropy in adult mouse ventricular myocytes.
Experimental approach: Cell shortening and Ca^{2+} transients were simultaneously recorded from isolated mouse ventricular myocytes loaded with the Ca^{2+}-sensitive fluorescent dye indo-1.
Key results: ET-1 decreased cell shortening in a concentration-dependent manner (pD_2 value of 10.1). The ET-1-induced decrease in cell shortening was associated with a decrease in Ca^{2+} transients. In addition, the Ca^{2+} transient/cell-shortening relationship was shifted to the right by ET-1, indicating decreased myofilament Ca^{2+} sensitivity. The instantaneous relationship of the rising phase of the Ca^{2+} transient and cell shortening was shifted to the right by ET-1. Decreased Ca^{2+} transients and cell shortening induced by ET-1 were markedly attenuated by the specific Na^+/Ca^{2+} exchange inhibitor SEA0400.
Conclusions and implications: ET-1-induced negative inotropy in mouse ventricular myocytes was mediated by decreased Ca^{2+} transients and myofilament Ca^{2+} sensitivity. These data are entirely consistent with the involvement of increased Ca^{2+} extrusion via the Na^+/Ca^{2+} exchanger in the ET-1-mediated decrease in Ca^{2+} transients. Decreased Ca^{2+} sensitivity may be due to retardation of cell shortening in response to a rise in Ca^{2+} transients.
British Journal of Pharmacology (2007) **152**, 456–463; doi:10.1038/sj.bjp.0707392; published online 16 July 2007

Keywords: Ca^{2+} transient; endothelin-1; mouse ventricular myocytes; Na^+/Ca^{2+} exchanger; negative inotropic effect; SEA0400

Abbreviations: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; ET-1, endothelin-1; Indo-1/AM, acetoxymethylester of indo-1; NCX, Na^+/Ca^{2+} exchanger; NIE, negative inotropic effect; PIE, positive inotropic effect; SR, sarcoplasmic reticulum

Introduction

While novel gene technology has mostly been applied to mice to elucidate the functional role of cardiovascular regulatory proteins and/or to create models of cardiovascular diseases, the cardiovascular regulatory process has not yet been well established in this species. Since the findings arising from gene-manipulated pathological models in mice need to be adapted to human patients, it is crucial to clarify any similarities and differences between the cardiovascular regulatory system in mice and larger mammalian species. The most prominent difference in the endothelin (ET) receptor-mediated cardiac contractile regulation is that ET-1 exclusively elicits a sustained negative inotropic effect (NIE) in mouse ventricular myocytes (Sekine *et al*., 1999; Sakurai *et al*., 2002). This contrasts with myocytes from larger mammalian species, in which ET-1 exclusively induces a positive inotropic effect (PIE) or a biphasic effect (a transient NIE proceeding to a sustained PIE) (Takanashi and Endoh, 1992; Endoh *et al*., 1998; Woo and Lee, 1999). Since ET-1 may play a key role in the progression of cardiovascular disorders, including congestive heart failure and ischemic heart disease (Wei *et al*., 1994; Sakai *et al*., 1996; Giannessi *et al*., 2001; Komuro, 2001), it is important to elucidate the mechanisms involved in the species-dependent differences in contractile regulation, namely in the NIE of ET-1 in mouse cardiomyocytes. In the present study, we characterized the ET-1-induced NIE, and analysed pharmacologically the NIE mechanism, by simultaneously measuring cell shortening and Ca^{2+} transients in indo-1-loaded mouse single ventricular myocytes.

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Received 14 February 2007; revised 9 April 2007; accepted 18 June 2007; published online 16 July 2007

Methods

Cell isolation and acetoxymethylester of indo-1 loading
All experiments were performed in accordance with the 'Guiding Principles for the Care and Use of Laboratory Animals' approved by The Japanese Pharmacological Society. Ventricular cardiomyocytes were isolated from male C57BL mouse (6–10 weeks old) hearts by Langendorff perfusion and collagenase treatment, as described previously (Nishimaru *et al*., 2001). Myocytes were loaded with indo-1, a Ca^{2+}-sensitive fluorescent dye, by incubation with nominally Ca^{2+}-free HEPES/Tyrode solution containing 5 μM acetoxymethylester of indo-1 (indo-1/AM) for 30 min at room temperature. After loading, the myocytes were plated in a superfusion chamber and superfused with nominally Ca^{2+}-free HEPES/Tyrode solution for at least 30 min, to wash out extracellular dye and allow de-esterification of intracellular indo-1. The HEPES/Tyrode solution contained (in mM): NaCl, 135; KCl, 5.4; $MgCl_2$, 1; $CaCl_2$, 1.8; NaH_2PO_4, 0.33; HEPES, 5; and glucose, 5.5 (pH 7.4), and was equilibrated with 100% O_2.

Measurements of cell shortening and Indo-1 fluorescence ratio
The chamber was placed on the stage of an inverted microscope (Diaphot TMD 300; Nikon, Tokyo, Japan) adapted for epifluorescence measurements. The cells were superfused with HEPES/Tyrode solution at a rate of 3 ml min^{-1} at 33°C, and stimulated electrically by square-wave pulses with a voltage ~30–40% above the threshold, at a frequency of 0.5 Hz. In basal conditions of 1.8 mM extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$), average cell shortening was 7.72±0.36% of the diastolic cell length ($n=47$).

The fluorescence of indo-1 was excited with light from a xenon lamp at a wavelength of 355 nm, reflected by a 380 nm long-pass dichroic mirror and detected by a fluorescence spectrophotometer (CAM-230; Japan Spectroscopic Co., Tokyo, Japan). Excitation light was applied to myocytes through a neutral-density filter to minimize the photobleaching of indo-1. The emitted fluorescence was collected by an objective lens (CF Fluor DL40; Nikon, Japan) and then separated by a 580 nm long-pass dichroic mirror (Omega Optical, Brattleboro, VT, USA), after passing through a 380 nm long-pass dichroic mirror. The fluorescence light was subsequently split with a 425 nm dichroic mirror to permit simultaneous measurements of light at both 405 and 500 nm wavelengths through band-pass filters. The emission field was restricted to a single cell with the aid of an adjustable window. The fluorescence ratio (405/500 nm) was used as an indicator of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Grynkiewicz *et al*., 1985).

Cells were simultaneously illuminated with red light (>620 nm) through the normal bright-field illumination of the microscope, and the bright-field images of one myocyte were collected by an objective lens and separated first by a 580 nm long-pass dichroic mirror (Omega Optical). The bright-field cell images were projected onto the photodiode array of an edge detector (C6294-01; Hamamatsu Photonics, Hamamatsu, Japan) with 5 ms temporal resolution, and the cell length was monitored by images simultaneously with fluorescent dye indo-1.

Statistics
All values are expressed as means±s.e. The statistical significance of differences between means was evaluated by one-way or two-way analysis of variance (ANOVA), or by Student's *t* test.

Chemicals
The drugs and reagents used were ET-1 (Peptide Institute, Osaka, Japan), nicardipine hydrochloride (Sigma, St. Louis, MO, USA); indo-1/AM (Molecular Probes, Eugene, OR, USA); collagenase (Yakult, Tokyo, Japan). SEA0400 (2-(4-((2,5-difluorophenyl)methoxy)phenoxy)-5-ethoxyaniline) was provided by Taisho Pharmaceutical Company, Ltd. Other reagents used were of the highest-grade purity that was commercially available.

Results

Effects of ET-1 on cell shortening and Ca^{2+} transients
We first determined the efficacy and potency of ET-1 in mouse ventricular myocytes. ET-1 decreased the cell shortening in a concentration-dependent manner. The NIE was induced at concentrations of $\geq 10^{-11}$ M, with the maximum inhibition being achieved at 10^{-8} M. A concentration–response curve for the decrease in cell shortening is shown in Figure 1. The maximum inhibition of cell shortening induced by ET-1 at 10^{-8} M amounted to 77.4±8.8% of the basal cell shortening (defined as 100%), as shown in Figure 1b. The pD_2 value and Hill slope were 10.1±0.2 and −1.2±0.4, respectively ($n=5$).

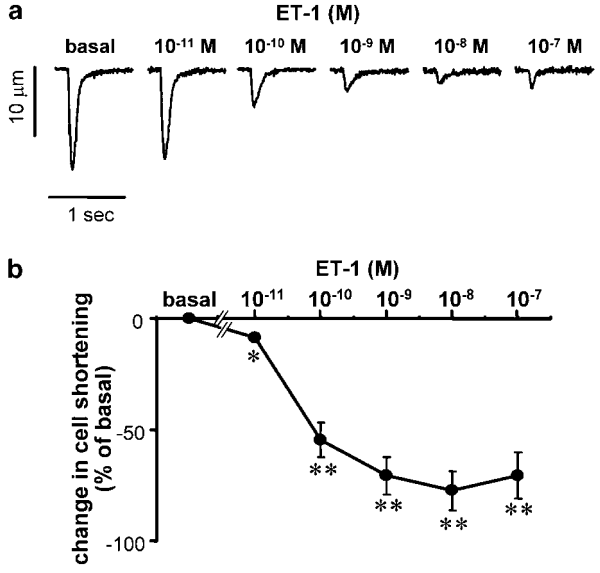

Figure 1 Effects of ET-1 on cell shortening in adult mouse ventricular myocytes. (**a**) Representative tracings of cell shortening in the absence and presence of increasing concentrations of ET-1. Individual tracings were obtained by means of signal averaging of 10–20 successive signals. (**b**) Summarized concentration–response relationship for the effect of ET-1 on cell shortening. Symbols with bars indicate the mean±s.e.m. from six experiments. *$P<0.05$ and **$P<0.01$ versus the value in the absence of ET-1. ET-1, endothelin-1.

Figure 2 illustrates typical tracings (Figure 2a) and the summarized data (Figure 2b) of the effects of ET-1 on cell shortening and Ca^{2+} transients (indo-1 fluorescence ratio) in adult mouse ventricular myocytes. The effects of two concentrations (10^{-10} and 10^{-9} M) of ET-1 were investigated in each myocyte, since long-term photoexcitation for the detection of indo-1 fluorescence causes cell damage. In the presence of 10^{-10} and 10^{-9} M ET-1, cell shortening was significantly decreased. When myocytes were exposed to 10^{-9} M ET-1, the cell shortening started to decrease within 2 min, reached a steady level within 5–7 min and remained at a stable, decreased level thereafter. The ET-1-induced effect on cell shortening was associated with a decrease in Ca^{2+} transients, indicating that the ET-1-induced NIE was mediated, at least in part, by a decrease in Ca^{2+} transients. In the presence of 10^{-10} and 10^{-9} M ET-1, Ca^{2+} transients were decreased (Figure 2b) and these changes in Ca^{2+} transients were statistically significant at both concentrations of ET-1, but they were relatively small compared with the pronounced decrease in cell shortening.

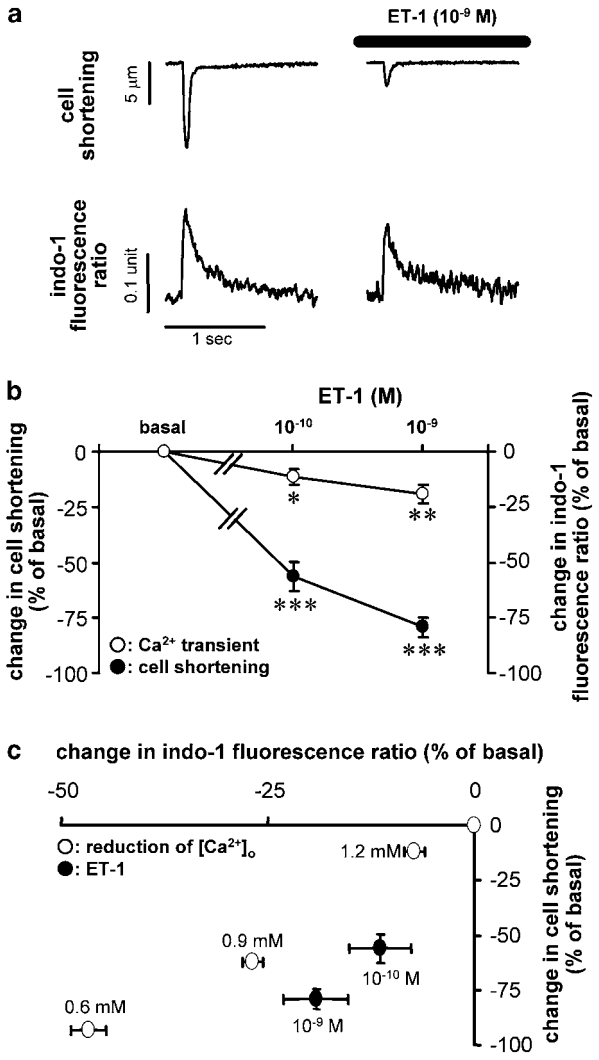

Figure 2 Effects of ET-1 on cell shortening and Ca^{2+} transients in adult mouse ventricular myocytes. (**a**) Representative recordings of cell shortening (upper tracings) and Ca^{2+} transients (lower tracings) in the absence (left) and presence (right) of ET-1 (10^{-9} M). Individual tracings were obtained by means of signal averaging of 30 successive signals. (**b**) Summarized data on the effects of ET-1 on cell shortening and Ca^{2+} transients. Symbols with bars indicate the mean ± s.e.m. from nine experiments. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ versus the value in the absence of ET-1. (**c**) Difference in the relationship between Ca^{2+} transients (indo-1 fluorescence ratio) and cell shortening induced by reduction of extracellular Ca^{2+} concentration or application of ET-1. Symbols with bars indicate the mean ± s.e.m. from five to nine experiments. ET-1, endothelin-1.

Effects of ET-1 on myofilament Ca^{2+} sensitivity

To investigate whether ET-1 changes the myofilament Ca^{2+} sensitivity, two series of experiments were carried out. We first compared the relationship between the amplitude of Ca^{2+} transients and cell shortening during changes in $[Ca^{2+}]_o$ and during exposure to ET-1. With stepwise decreases in $[Ca^{2+}]_o$ from 1.8 to 1.2, 0.9 and 0.6 mM, cell shortening and Ca^{2+} transients decreased in a concentration-dependent manner. Figure 2c shows the relationship between the amplitude of Ca^{2+} transients and cell shortening during exposure to ET-1 or reduction in $[Ca^{2+}]_o$. There was no significant difference in basal cell shortening and indo-1 fluorescence ratio between these two groups (data not shown). The relationship during induction of the NIE by ET-1 was shifted to the right of the relationship for a reduction in $[Ca^{2+}]_o$ (Figure 2c), suggesting that ET-1 may decrease the myofilament Ca^{2+} sensitivity, in addition to decreasing Ca^{2+} transients.

In a second series of experiments, the instantaneous relationship between the indo-1 fluorescence ratio and cell shortening was examined. The decrease in cell shortening induced by 10^{-10} M ET-1 was reversed to the control level before exposure to ET-1 by raising $[Ca^{2+}]_o$ from 1.8 to 3.6 mM (Figure 3). During application of 10^{-10} M ET-1, doubling of $[Ca^{2+}]_o$ reversed the decreased cell shortening induced by ET-1, to the basal level shown in Figure 3a and summarized in Figure 3b. On average, cell shortening was reversed to 99.2 ± 6.7% of basal cell shortening ($n=5$), whereas the amplitude of Ca^{2+} transients with ET-1 at 3.6 mM $[Ca^{2+}]_o$ was significantly higher than the basal level (Figure 3b; 121.7 ± 5.8% of basal Ca^{2+} transients), indicating an ET-1-induced decrease in Ca^{2+} sensitivity. The trajectory that reflects the instantaneous relationship of cell shortening and Ca^{2+} transients during the course of twitch contractions, in the presence of ET-1 at 3.6 mM $[Ca^{2+}]_o$, was compared with that of the control relationship at 1.8 mM $[Ca^{2+}]_o$. The phase–plane diagrams of the time-dependent changes in cell shortening and Ca^{2+} transients indicate that the trajectory was shifted to right with ET-1 at 3.6 mM $[Ca^{2+}]_o$, indicating that ET-1 decreased myofilament Ca^{2+} sensitivity. Furthermore, detailed inspection of the changes in phase–plane trajectory reveals that, in the rising phase, cell shortening was prominently retarded, while in the relaxation phase, the trajectory was actually superimposable on that of the control trajectory (Figure 3c).

Pharmacological modulation of the ET-1-induced effects on cell shortening and Ca^{2+} transients

The next series of experiments were performed to obtain an insight into the contribution of $[Ca^{2+}]_i$-regulatory systems,

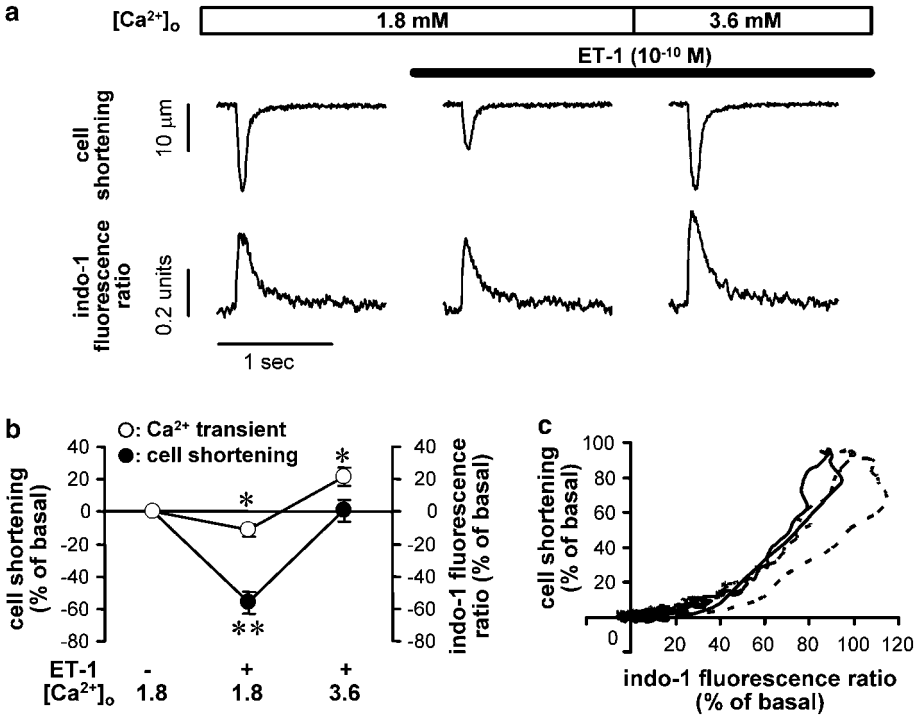

Figure 3 Effects of ET-1 on cell shortening and Ca^{2+} transients, and the instantaneous relationship of cell shortening versus indo-1 fluorescence signal in adult mouse ventricular myocytes. (**a**) Representative tracings of cell shortening (upper tracings) and Ca^{2+} transients (lower tracings) in the absence of ET-1 (left), in the presence of ET-1 (10^{-10} M, middle) and doubling of $[Ca^{2+}]_o$ in the presence of ET-1 (right). Individual tracings were obtained by means of signal averaging of 30 successive signals. (**b**) Summarized data on cell shortening (closed circles) and Ca^{2+} transients (open circles). Symbols with bars indicate the mean ± s.e.m. from six experiments. *$P<0.05$ and **$P<0.01$ versus the value in the absence of ET-1. (**c**) Instantaneous plots of cell shortening versus indo-1 fluorescence ratio in the absence of ET-1 ($[Ca^{2+}]_o = 1.8$ mM, solid line) and in the presence of ET-1 ($[Ca^{2+}]_o$ doubled to 3.6 mM, dashed line). $[Ca^{2+}]_o$, extracellular Ca^{2+} concentration; ET-1, endothelin-1.

such as the L-type Ca^{2+} channel and/or the Na^+/Ca^{2+} exchanger (NCX), to the ET-1-induced changes in Ca^{2+} signalling. In the presence of nicardipine (10^{-6} M), an L-type Ca^{2+} channel antagonist, cell shortening and Ca^{2+} transients were decreased to a moderate extent by 33.5 ± 2.4 and 13.3 ± 3.0% of the basal levels, respectively ($n = 5$). ET-1 (10^{-9} M) further decreased cell shortening and Ca^{2+} transients in the presence of nicardipine, as illustrated in Figure 4a and summarized in Figure 4c ($n = 5$). To examine whether there was an interaction between nicardipine and ET-1 in the inhibition of cell shortening and Ca^{2+} transients, that is, whether inhibition induced by both agents is more or less than additive, two-way ANOVA was carried out. The test revealed that the ET-1-induced effects were significantly augmented in the presence of nicardipine.

SEA0400 (10^{-6} M), a selective NCX inhibitor (Tanaka *et al*., 2002), increased cell shortening and Ca^{2+} transients, as shown in Figure 4b (middle panel), by 48.4 ± 14.7 and 21.8 ± 15.5% of the basal levels, respectively ($n = 6$). In the presence of SEA0400 (10^{-6} M), the ET-1 (10^{-9} M)-induced decrease in cell shortening was markedly attenuated, as shown in Figure 4b (right panel) and summarized in Figure 4c (left panel). The effects of ET-1 on Ca^{2+} transients were correspondingly reversed by pre-exposure to SEA0400 (Figure 4c, right panel). These results indicated that the NCX might play an important role in ET-1-induced decrease in Ca^{2+} transients and the subsequent NIE.

A series of experiments was carried out to examine whether the inhibitory action of SEA0400 was specific for the inhibition of NCX activity or could be observed in general when cell shortening and Ca^{2+} transients were increased by any other intervention. For this purpose, we investigated the influence of increasing $[Ca^{2+}]_o$ to 2.7 mM, which increased cell shortening and Ca^{2+} transients to an equivalent extent to that induced by SEA0400. An elevation of $[Ca^{2+}]_o$ from 1.8 to 2.7 mM increased cell shortening and Ca^{2+} transients by 60.3 ± 17.9 and 10.4 ± 3.6% of control values, respectively ($n = 6$), which were comparable to those induced by 10^{-6} M SEA0400. At 2.7 mM $[Ca^{2+}]_o$, ET-1 (10^{-9} M) decreased cell shortening and Ca^{2+} transients by 63.9 ± 8.7 and 18.4 ± 4.6% of the respective basal levels ($n = 6$), which were not significantly different from the effects induced by ET-1 at 1.8 mM $[Ca^{2+}]_o$ (78.6 ± 4.9 and 17.7 ± 5.4%; $n = 7$). These findings imply that the PIE induced by SEA0400 does not contribute to the inhibitory action of the compound.

Discussion

In mouse ventricular myocytes, ET-1 elicited a sustained NIE in a concentration-dependent manner, without inducing a PIE. This is in strong contrast to the inotropic response to ET-1 in ventricular myocardium from larger mammalian species, because in most of these species ET-1 induces a PIE or

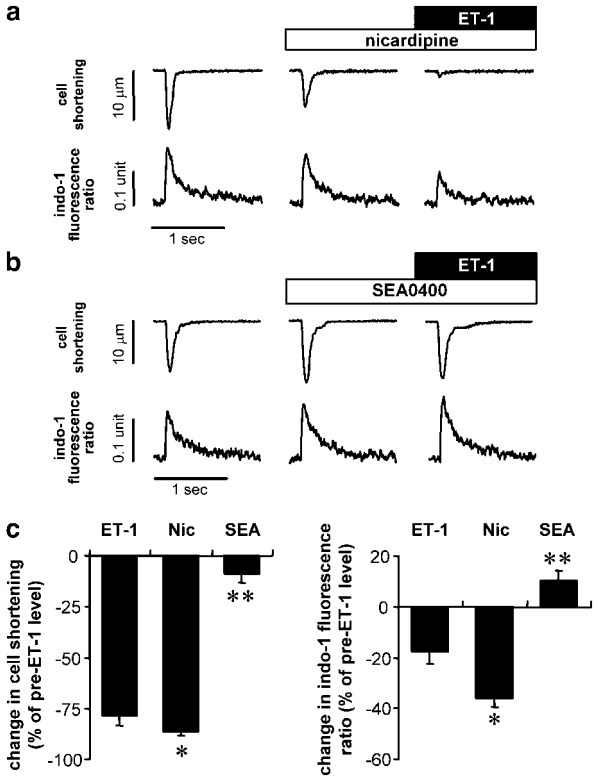

Figure 4 Influence of nicardipine or SEA0400 on basal cell shortening and Ca^{2+} transients, and on the effects of ET-1 in adult mouse ventricular myocytes. (**a**) Representative tracings of cell shortening (upper tracings) and Ca^{2+} transients (lower tracings) before application of nicardipine (left), and after application of 10^{-6} M nicardipine in the absence of ET-1 (middle) and in the presence of 10^{-9} M ET-1 (right). Individual tracings were obtained by means of signal averaging of 30 successive signals. (**b**) Representative tracings of cell shortening (upper tracings) and Ca^{2+} transients (lower tracings) before application of SEA0400 (left), and after application of 10^{-6} M SEA0400 in the absence of ET-1 (middle) and in the presence of 10^{-9} M ET-1 (right). Individual tracings were obtained by means of signal averaging of 25 successive signals. (**c**) Summarized data of the effects of ET-1 in the absence or presence of nicardipine or SEA0400 on cell shortening (left) and Ca^{2+} transients (right). Bars indicate the mean$\pm$s.e.m. from five to nine experiments. *$P<0.05$ and **$P<0.01$ versus controls. Statistical analysis was carried out by means of two-way analysis of variance. ET-1, endothelin-1; SEA0400; 2-(4-((2,5-difluorophenyl)methoxy)phenoxy)-5-ethoxyaniline.

biphasic effect, that is, a sustained PIE subsequent to a transient NIE (for example, Takanashi and Endoh, 1991). The important finding in the present study is that the NIE of ET-1 was mediated by at least two cellular mechanisms in mouse ventricular myocytes, a decrease in Ca^{2+} transients and a decrease in myofilament Ca^{2+} sensitivity.

Mechanism of the ET-1-induced decrease in Ca^{2+} transients

The decrease in Ca^{2+} transients caused by ET-1 was antagonized by SEA0400, an NCX inhibitor. These data are entirely consistent with the involvement of increased Ca^{2+} extrusion via the NCX in the ET-1-mediated decrease in Ca^{2+} transients. ET-1-induced enhancement of cardiac NCX activity has been shown to result in an increase in Ca^{2+} transients in other mammalian species, including rats (Ballard and Schaffer, 1996), guinea pigs (Zhang *et al*., 2001) and rabbits (Yang *et al*., 1999), in which ET-1 induces a PIE (Takanashi and Endoh, 1991). These observations imply that the difference exists in cardiac excitation–contraction coupling in myocytes from mice and larger mammalian species, which may be responsible for a decrease or increase in Ca^{2+} transients produced by the ET-1-induced enhancement of NCX activity and/or L-type Ca^{2+} channels.

It is probable that the activation of NCX facilitates Ca^{2+} influx under certain experimental conditions in mice, as in other species, namely, because the relatively high resting intracellular Na^+ concentration ($[Na^+]_i$) in rodents (Shattock and Bers, 1989) is favourable for an increase in Ca^{2+} influx via NCX activity. In fact, Terracciano *et al*. (1998) have shown the presence of Ca^{2+} influx through NCX at rest in mouse ventricular myocytes. However, it should be noted that, in these experiments, the Ca^{2+} influx via NCX occurred after a long rest period under conditions that are highly favourable for Ca^{2+} influx via NCX activity. Since the Ca^{2+} uptake function of sarcoplasmic reticulum (SR) is highly developed in the mouse heart (Tanaka *et al*., 1998), $[Ca^{2+}]_i$ may become very low during rest periods, as an increase in Ca^{2+} influx occurs readily via NCX in the presence of relatively high $[Na^+]_i$.

In the present study, the inhibition of NCX induced by SEA0400 had an effect on cell shortening and Ca^{2+} transients opposite to that of ET-1, supporting the view that Ca^{2+} efflux via NCX predominates during the cardiac cycle and that the activation of NCX by ET-1 produces an NIE in mouse ventricular myocytes. It is noteworthy that mouse ventricular myocytes have an extremely short action potential (Tanaka *et al*., 1998). Therefore, it is reasonable to postulate that the membrane potential is more negative than the equilibrium potential of the forward mode of NCX for a relatively longer period during the cardiac cycle, which favours Ca^{2+} extraction via NCX activation. It has been shown that SEA0400 at a concentration of 10^{-6} M, as used in the present study, inhibits cardiac NCX activity by $>80\%$, but has no effect on other cardiac ion channels (Tanaka *et al*., 2002) or on myofilament Ca^{2+} sensitivity (Tanaka *et al*., 2005). Experimental evidence implies that, as an NCX inhibitor, SEA0400 is highly specific and more potent than another NCX inhibitor, KB-R7943, which has been applied extensively to cardiac muscle, but which has an inhibitory action on many other cardiac ion channels (Tanaka *et al*., 2002).

Stimulation of cardiac α_1-adrenoceptors that share the subcellular signalling mechanisms with ET-receptor activation elicits, likewise, an NIE in association with a decrease in Ca^{2+} transients that is susceptible to SEA0400 in mouse cardiomyocytes (Nishimaru *et al*., 2001; Tanaka *et al*., 2005). These observations indicate that the activation of NCX also occurs via α_1-adrenoceptor stimulation, leading to an NIE in mouse ventricular myocytes.

It should be noted that the present study was performed at 33°C, while previous experiments were carried out at room temperature (22–23°C), in which the NIE of ET-1 was associated with an insignificant decrease in Ca^{2+} transients in mouse ventricular myocytes (Sakurai *et al*., 2002). Because NCX activity is highly sensitive to temperature (Kimura

et al., 1987), the decrease in NCX activity at low temperature might have led to an underestimation of the ET-1-induced decrease in Ca^{2+} transients in previous experiments. A similar temperature-dependence has been reported for the α-adrenoceptor-mediated NIE in mouse ventricular myocardium (Nishimaru *et al*., 1999).

Mechanism of ET-1-induced decrease in myofilament Ca^{2+} sensitivity

The ET-1-induced rightward shift of the relationship between Ca^{2+} transients and cell shortening during reduction of $[Ca^{2+}]_o$ (Figure 2c) indicates strongly that ET-1 decreased myofilament Ca^{2+} sensitivity in mouse ventricular myocytes. This is in sharp contrast to the increase in Ca^{2+} sensitivity, generally observed by exposure to ET-1 in ventricular myocardium of other mammalian species (Wang *et al*., 2000, 1991).

It has been reported that ET-1 generates reactive oxygen species in myocytes (Daou and Srivastava, 2004), which leads to an irreversible decrease in myofilament Ca^{2+} sensitivity (Gao *et al*., 1996). Such a mechanism may not be responsible for the ET-1-induced decrease in Ca^{2+} sensitivity in mouse ventricular myocytes, as the decrease in Ca^{2+} sensitivity is transient, that is, reversible. The phase–plane trajectory reveals that, during the rising phase of cell shortening, a pronounced dissociation of contractile function from $[Ca^{2+}]_i$ occurred, whereas in the relaxation phase, the trajectory was actually superimposable on that of the control cells in the absence of ET-1 (Figure 3c). These findings imply that the ET-1-induced modulation of Ca^{2+} sensitivity is dependent on the contraction phase, and is due to the reversible kinetic property that induces an apparent decrease in Ca^{2+} sensitivity. When the steady-state $[Ca^{2+}]$–cell shortening relationship was examined under tetanic stimulation induced by high-frequency electrical stimulation, in the presence of cyclopiazonic acid that inhibits Ca^{2+} uptake of the SR, the Ca^{2+} sensitivity at steady state was actually found to be increased by ET-1. However, the development of shortening was markedly retarded, causing an apparent decrease in Ca^{2+} sensitivity in mouse ventricular myocytes (Nishimaru and Endoh, 2005). A decrease in actomyosin ATPase activity induced by ET-1 (McClellan *et al*., 1996) may partly contribute to the decrease in Ca^{2+} sensitivity, but the exact mechanism remains to be clarified.

Role of L-type Ca^{2+} channels in ET-1-induced contractile effects

In mammalian ventricular myocardium, the effects of ET-1 on L-type Ca^{2+} current are complex and controversial. ET-1 enhances L-type Ca^{2+} current in rat and rabbit ventricular myocytes (Lauer *et al*., 1992; He *et al*., 2000), while it suppresses the current in guinea pig and rabbit myocytes (Tohse *et al*., 1990; Xie *et al*., 1996; Watanabe and Endoh, 1999). In addition to species-dependent differences, experimental procedures may also contribute to the controversy surrounding the findings. Certain regulatory proteins may be lost by application of conventional patch clamp techniques, which may be overcome by means of the perforated patch clamp technique (He *et al*., 2000). In addition, during β-adrenoceptor stimulation, ET-1 elicits a pronounced inhibition of the Ca^{2+} current by antagonizing the cAMP-mediated increase in Ca^{2+} current (Xie *et al*., 1996; Watanabe and Endoh, 1999, 2000).

It has been reported that the contractile activity of mouse ventricular myocardium is relatively resistant to L-type Ca^{2+} channel antagonists (Tanaka *et al*., 1998). In the present study, the ET-1-induced decrease in cell shortening and Ca^{2+} transients was significantly augmented in the presence of nicardipine, suggesting ET-1-induced activation of L-type Ca^{2+} channel activity. Nicardipine, at the concentration of 10^{-6} M used in the present study, is not highly selective for L-type Ca^{2+} channels (Hatano *et al*., 2003). However, it was important in the present experiments to employ a Ca^{2+} antagonist with less light-sensitivity, because of the necessity for light exposure for the detection of Ca^{2+} transients using the Ca^{2+}-sensitive fluorescence probe indo-1. While nicardipine has some degree of light-sensitivity, it is much less sensitive than more selective Ca^{2+} antagonists, such as nifedipine and nisoldipine. Furthermore, it has been demonstrated that the Ca^{2+} channel blocking activity of nicardipine is not modulated by the light at 355 nm, which is used for detection of indo-1 fluorescence signals (Sanguinetti and Kass, 1984). The concentration of nicardipine (10^{-6} M) was chosen by the extent of the decreases in cell shortening and Ca^{2+} transients in mouse ventricular myocytes in preliminary experiments, and nicardipine at 10^{-6} M decreased cell shortening and Ca^{2+} transients by 33.5 and 13.3%, respectively. This may not be large enough, but just sufficient to examine the role of L-type Ca^{2+} channels in the ET-1-induced NIE. The potential contribution of non-selective effects of nicardipine, however, could not be completely excluded in the current study.

ET-1 decreases repolarizing K^+ currents to prolong action potential duration, resulting in secondary increases in Ca^{2+} influx through L-type Ca^{2+} channels in rat ventricular myocytes (Damron *et al*., 1993). In mouse ventricular myocytes, however, ET-1-induced regulation of K^+ currents remains to be demonstrated.

Limitation of the current study

It should be noted that the experimental conditions employed in the current study were quite different from those under which the heart acts *in vivo* in mice, including heart rate and body temperature. While the present findings indicate that the alteration in contractility and Ca^{2+} transients in response to ET-1 in mouse ventricular myocytes is quite different from that in myocytes from larger mammalian species observed under identical experimental conditions, further study is required to establish the contractile regulation by ET in mice, taking further multiple aspects into consideration. By comparison with the effect of elevation of $[Ca^{2+}]_o$, it was clear that the inhibitory action of SEA0400 on the NIE of ET-1 was not due to the physiological antagonism induced by its PIE. To establish the role of NCX in contractile regulation induced by ET-1, further study by means of rapid solution exchange devices (such as those described by Levi *et al*., 1996) and by use of caffeine to exclude the contribution of SR Ca^{2+} ATPase is required.

Summary
The present study indicates that the ET-1-induced NIE in adult mouse ventricular myocytes is mediated partly by a decrease in Ca^{2+} transients and myofilament Ca^{2+} sensitivity. Our findings suggest a role for enhanced Ca^{2+} extrusion via forward-mode NCX in the ET-1-induced decrease in Ca^{2+} transients. Further studies are required to establish the role of Na^{+}/Ca^{+} exchange in the responses to ET-1 of mouse cardiomyocytes. In addition, these differences between mice and larger mammalian species have to be taken into consideration when the mouse is employed as a genetic model of cardiovascular diseases.

Acknowledgements

This study was supported by a Grant-in-Aid for Encouragement of Young Scientists (No. 16790149) (KN) and for Scientific Research (B) (No. 16390064) (ME) from the Ministry of Education, Science, Sports, and Culture, Japan, and grants provided by the Uehara Memorial Foundation (KN). We thank Mr Ikuo Norota for his continuous technical advice.

Conflict of interest

The authors state no conflict of interest.

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