# Spinal Cord Injury Blunted Effects of Endothelin-1 on $Ca^{2+}$ Transients and Calcium Current in Isolated Rat Cardiomyocytes

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Short title: Effects of ET-1 on $Ca^{2+}$ Transients and Calcium Current

## Summary

**AIM:** Plasma endothelin-1 (ET-1) levels are elevated in spinal cord injury (SCI), and ET-1 may be involved in the pathophysiology of this condition. However, its effects on contractile function of the SCI rat heart are still unknown. To more clearly define the potential role of ET-1 following SCI, we investigated the effect of ET-1 on the contraction, calcium transients and L-type calcium current ($I_{Ca,L}$) in the control and SCI rat cardiomyocytes. **METHODS**: Sixteen Sprague-Dawley male rats aged 80-100 d and weighing 250-350 g were randomized into control and SCI groups. Fourteen days following compression injury to the spinal cord, effects of ET-1 on the contraction,calcium transients and $I_{Ca,L}$ were studied in the control and SCI rat cardiomyocytes by the technique of simultaneous measurement of intracellular $Ca^{2+}$ and contraction and by whole-cell configuration of the patch-clamp technique. **RESULTS**: In myocytes from control rats, ET-1 significantly increased contraction, the magnitude of $Ca^{2+}$ transients and the peak amplitude of $I_{Ca,L}$. However, ET-1 had little effect on the amplitude of contraction, calcium transients and $I_{Ca,L}$ in myocytes from SCI rats. **CONCLUSIONS**: These results suggest that the positive inotropic effects of ET-1 on control myocardial contraction may be altered in pathological states such as SCI.

**Key words** : Myocytes; Endothelin; Calcium transients; L-type calcium current; Rats

# Introduction

Endothelin-1 (ET-1) is a 21-amino acid peptide originally isolated from porcine aortic endothelial cells as a vasoconstricting agent (Yanagisawa *et al*. 1988). In addition to its potential vasoconstrictive effects, ET-1 produces a positive inotropic effect on the intact left ventricular (LV) and isolated myocytes from normal animals (Chu *et al*. 2005, Cingolani *et al*. 2006, De *et al*. 2008, Mebazaa *et al*. 1993). Recent studies suggest that the effects of ET-1 on normal myocardial contraction may be altered in pathologic states (Brás-silva *et al*. 2008, Cheng *et al*. 1996, Ito *et al*. 1997, Spinale *et al*. 1997). For example, Sakai and colleagues reported that following coronary artery occlusion in rats, the elevated plasma levels of ET-1 and myocardial accumulation of ET-1 were associated with a decline in LV pressure development (Sakai *et al*. 1996). Treatment with ET-1 receptor blocker has been shown to improve cardiac function in congestive heart failure (CHF) and myocardial ischemia (Cheng *et al*. 1996, Kiowski *et al*. 1995, Spinale *et al*. 1997). In CHF, ET-1 produces a direct depression of cardiomyocyte contractile performance that is associated with a significant decrease in the peak $Ca^{2+}$ transient (Suzuki *et al*. 1998).

Cardiovascular abnormalities after spinal cord injury (SCI) have been well documented in clinical studies (Collins *et al*. 2006, Myers *et al*. 2007, Teasell *et al*. 2000). The initial period after SCI is characterized by low arterial blood pressure and persistent bradycardia (Collins *et al*. 2006, Furlan *et al*. 2008). With time, blood pressure control becomes volatile and can be characterized by episodes of extreme

hypertension accompanied by pounding headache, slow heart rate, and upper body flushing. Individuals with spinal cord injury are reported to be at increased risk of mortality compared to able-bodied persons, and death among individuals with SCI is primarily due to cardiovascular abnormities (Devivo *et al*. 1992). However, the reasons for this are uncertain. One possibility is that SCI increases the risk of unstable blood pressure and cardiac arrhythmias or other electrocardiographic abnormalities that may predispose patients to adverse cardiac events (Leaf *et al*. 1993, Prakash *et al*. 2002).

Spinal cord injury has been shown to induce an immediate increase in plasma ET-1 levels and a sustained increase in tissue ET-1 levels (Salzman *et al*. 1996, Uesugi *et al*. 1996, Peters *et al*. 2003). Recent experimental and clinical studies indicated a role for ET-1 in the pathophysiology of SCI (Ogawa *et al*. 2008, Salzman *et al*. 1996, Uesugi *et al*. 1998). To more clearly define the potential role of ET-1 on myocytes following SCI, we investigated the effect of ET-1 on contraction, $Ca^{2+}$ transient, and L-type calcium current ($I_{Ca,L}$) in rat ventricular myocytes.

## Materials and Methods

This study was performed according to the guidelines for the care and use of laboratory animals published by U.S. National Academy Press in 1996 and the Guidelines for Animal Experiments of the Second Military Medical University of CPLA.

***SCI model in rats***

Sixteen Sprague-Dawley (SD) male rats aged 80-100 d and weighing 250-350 g were randomized into two groups. Group 1: Sham group (n=8). Group 2: Spinal cord injury (SCI) group (n=8).The SCI model in rats has been detailed previously (Liu *et al*. 1997,Xu *et al*. 1991). Briefly, male rats were subjected to chloral hydrate anesthesia (400 mg/kg, i.p.). After a T5–T6 laminectomy, SCI was induced using a New York University Impactor by dropping a 10 g weight. Animals subjected to identical surgical procedures, without impaction, served as sham operated controls.

***Cell Isolation***

Fourteen days after surgical procedures, single ventricular myocytes from the control and SCI group rats were isolated using an enzymatic dissociation procedure as described previously (Liu *et al*. 1995). Briefly, the animals were heparinized (500 IU/kg, i.p.) and were subjected to chloral hydrate anesthesia (400 mg/kg, i.p.). Hearts were removed quickly, and the aorta was cannulated and perfused by a langendorff perfusion apparatus with normal Tyrode's solution. The heart was then perfused with nominally $Ca^{2+}$-free Tyrode's solution for 5 minutes at a rate of 10 ml/min followed by perfusion with the same solution containing collagenase I (0.6mg/ml; sigma) at 37°C. Eight to ten minutes later, the ventricle was dissected, and cells were mechanically dispersed and stored in KB solution. This procedure consistently yielded an acceptable number of quiescent, relaxed ventricular cells. According to our procedure, we normally gained $10^6$ cells /heart and 3 cells from one heart were analyzed.

***Simultaneous measurement of intracellular $Ca^{2+}$ and contraction***

Myocytes were loaded with the fluorescent $Ca^{2+}$-sensitive indicator fura-2 by incubation with the membrane-permeable acetoxymethyl ester (AM) form of fura-2 (5 μM, Molecular Probes) for 30 min at room temperature. Myocytes were allowed to settle onto the coverslip that formed the base of the experimental chamber and were then superfused with Tyrode solution (see below for composition). The chamber was mounted on the stage of an inverted microscope (Nikon Diaphot) and fura-2 fluorescence was elicited by alternate illumination with 340 and 380 nm light obtained with a rotating filter wheel (Cairn Research Ltd.) mounted in front of a Xenon lamp. The fluorescence emitted at 510 nm was monitored using a photoincrease multiplier tube. The ratio of the fluorescence emitted at 510 nm during excitation at 340 nm to that emitted during excitation at 380 nm was obtained using an analog divide circuit and was used as a measurement of $[Ca^{2+}]i$. The amplitude of the $Ca^{2+}$ transient was measured as the difference between end diastolic $[Ca^{2+}]i$ and the $[Ca^{2+}]i$ at the peak of the rapidly rising phase of the $Ca^{2+}$ transient (Isenberg *et al*. 1994 ).

Contraction (cell shortening) was measured optically with a video edge detector system (Rotatech, Norwalk, CA, USA) (Steadman *et al*. 1988). The image of a contracting myocyte was detected by a CCD TV camera (TM 524, PULNiX, Hants, UK) on the side port of the microscope and the camera signal was then fed into the edge detector circuitry. The system functioned at the frame rate of the camera (50 Hz), and the accuracy with which the measurement cursors followed both ends of the

myocyte were verified continuously on a TV monitor. The frame rate is slow because our equipment is old, so there is a methodical limitation.

***Electrophysiological measurements***

Isolated rat ventricular myocytes were placed in a perfusion chamber and superfused with external solution at a flow rate of 1.5 ml/min. Patch electrodes were fabricated from borosilicate glass with a Micropipette Puller Model 97 (Sutter Instrument Company, California, USA). Its resistance was 2-4 MΩ when the electrode was filled with the internal solution. The potential of the electrode was adjusted to zero current between the internal solution and the external solution immediately before seal formation. After obtaining a gigaseal, a suction pulse was applied to establish the whole-cell mode. Series resistance was electronically compensated by 70–90%.

$I_{Ca,L}$ was elicited by depolarization to +10 mV for 400 ms, following a 300-ms-long prepulse to -40 mV from the holding potential of -80 mV at 15 s intervals. Any preparations showing insufficient voltage control were not included in the study. With $K^+$ currents blocked using the caesium electrode solution, and $Na^+$ currents inactivated at -40 mV, transient inward currents invoked by step depolarization from -40 to 10 mV were considered to be $I_{Ca,L}$(Dukes *et al*. 1991). This protocol elicited a time- and voltage-dependent current which had all the characteristics of $I_{Ca,L}$. These currents were completely blocked by verapamil. Membrane capacitance was measured using the calibrated capacity compensation circuit of the voltage clamp amplifier using a 5 mV hyperpolarizing pulse. We expressed all the current data as current density (pA/pF) by

normalizing the peak $I_{Ca,L}$ for each cell to cell capacitance. The current-voltage (I-V) relationship of $I_{Ca,L}$ was obtained by plotting the peak current amplitude in response to 400 ms voltage pulses from -50 to +60 mV with 10 mV increments every 15 s. In the present study, we studied the effects of 1nM, 5nM nad 10 nM ET-1 on $Ca^{2+}$ currents and found ET-1 dose-dependent increased $Ca^{2+}$ currents in myocytes of control rats and selected the dose of 5nM for the whole experiments

All experiments were performed at room temperature (20–23℃). Command pulses were delivered and data were acquired with a patch-clamp amplifier (EPC-10, HEKA Elektronik Dr. Schulze GmbH, Pfalz ,Germany) controlled by the PULSE software connected to a computer.

***Experimental Solutions***

The Tyrode's solution (as mmol/l): NaCl 135, KCl 5.4, $CaCl_2$ 1.2, $MgCl_2$ 1, $NaH_2PO_4$ 0.33, HEPES 5, and glucose 11 (pH adjusted to 7.4 with NaOH). Nominally $Ca^{2+}$-free Tyrode's solution was prepared by simply omitting $CaCl_2$. The KB solution (as mmol/l): KCl 30, L-glutamic acid 50, KOH 80, $KH_2PO_4$ 30, taurine 20, HEPES 10, EGTA 0.5, $MgSO_4$ 3 and glucose 10 (pH adjusted to 7.4 with KOH). The external solution (as mmol/l): NaCl 133.5, CsCl 4.0, $CaCl_2$ 1.8, $MgCl_2$ 1.2, Glucose 11.1, Hepes 10 (pH adjusted to 7.4 with NaOH). The internal solution (as mmol/l): CsCl 120, EGTA 10, $Na_2ATP$ 5, Hepes 10 (pH adjusted to 7.2 with CsOH).

***Statistical analysis***

Data were presented as means±SD and were analysed using analysis of variance followed by Student-Newman-Keuls *post hoc* test to assess the significance level. Differences between two groups were considered significant when $P < 0.05$.

## Results

### *Effects of ET-1 on $Ca^{2+}$ transients and contraction in myocytes from control and SCI group rats*

We first examined the characteristics of the effects of endothelin-1 at 5 nmol/l on the $Ca^{2+}$ transients and cell shortening in myocytes isolated from control rats. Fig.1 shows representative records of contraction and the associated $Ca^{2+}$ transients prior to and during application of ET-1 at 5 nmol/l for 10min in control ventricular myocytes loaded with fura-2. ET-1 at 5 nmol/l induced a significant increase in fura-2 ratio at 5 nmol/l (0.115±0.012 au vs 0.089±0.011 au, n=12, P<0.01) associated with an increase in cell shortening (7.13±0.78 μm vs 4.89±0.70 μm, n=12, P<0.01) (Fig 2). However, ET-1 at 5 nmol/l had little effect on the amplitude of contraction and calcium transients in myocytes from SCI rats (Fig.2, Fig.3).

### *Effect of ET-1 on $I_{Ca,L}$ in myocytes from control and SCI group rats*

Fig.4a shows a time course of the effect of 5 nmol/l ET-1 on peak $I_{Ca,L}$ density evoked by a step to +10 mV in a single myocyte of control or SCI rat .In control rat myocytes, application of 5 nmol/l ET-1 to the bath induced an increase in $I_{Ca,L}$ that

started in 3 min, reached a maximum value after 6–8 min, and reversed by washout. However, 5 nmol/l ET-1 did not increase $I_{Ca,L}$ significantly in SCI group myocytes. Fig.4b and 4c shows the average current density-voltage before and after administration of 5 nmol/l ET-1 in control and SCI rat myocytes respectively. A significant increase in current density was observed in the voltage range between -10 and +30 mV after application of ET-1 to the bath solution of control rat myocytes. $I_{Ca,L}$ at test potentials positive from -10 to +30 mV significantly increased. However, in SCI rat myocyets, ET-1 had little effect on $I_{Ca,L}$ (Fig.4c), which is consistent with the lack effect of ET-1 on contraction and calcium transients.

## Discussion

The present study studied the effects of ET-1 on the contraction, calcium transients and L-type calcium current in the control and SCI rat cardiomyocytes. We performed contractile force measurements and voltage-clamp analysis of membrane currents and experimental results shows that ET-1 significantly increased contractile force by enhancement of $Ca^{2+}$ influx through the L-type calcium channels in control rat myocytes. However, ET-1 had little effect on the amplitude of contraction, calcium transients and $I_{Ca,L}$ in myocytes of SCI rats.

It is generally accepted that calcium influx is the main factor eliciting cardiac contractions (Vites *et al*. 1996). Thus, increased $I_{Ca,L}$ might play the most important role in increasing cardiac contractility. The increase in $I_{Ca,L}$ induced by ET-1 may contribute

to an increase in $Ca^{2+}$ transients probably by activation of $Ca^{2+}$ release induced, by $Ca^{2+}$ and trigger positive inotropic action induced by ET-1. The positive inotropic effects of ET-1 on control myocytes are supposed to be induced by Gq protein-mediated activation of protein kinase C and a consequent variable combination of elevation of cytosolic calcium levels and myofilament sensitization to calcium (Sugden *et al*. 2003, Sugden *et al*. 2005).

Based on our present experimental findings, it is rather difficult to identify the mechanisms underlying the absence of the inotropic effect of ET-1 in SCI rat myocytes. It could be due to downregulation of ET-1 receptors. It has been reported that fourteen days following compression injury to the spinal cord, there was a significant upregulation in both the immunoexpression and number of astrocytes expressing the $ET_B$ receptor in both gray and white matter. Furthermore, there was a near disappearance of $ET_B$ receptor immunoreactivity in ependymal cells and $ET_A$ receptor immunoreactivity in primary afferent fibers (Peters *et al*. 2003). These results showed that there was upregulation or downregulation of ET-1 receptors in tissues after SCI. However, alterations in ET-1 receptor density or affinity have not been reported in SCI rat hearts. The absent inotropic effect of ET-1 in SCI rat myocytes also could be due to desensitization of ET-1 receptors, which might result in the lack effect of ET-1 on $I_{Ca,L}$ current and the consequent lack effect ET-1 on contraction.

ET-1 receptors can couple to Gq proteins and protein kinase C, thereby potentially exerting positive inotropic effects (Sugden *et al*. 2003, Sugden *et al*. 2005). ET-1 receptors can also couple to Gi proteins and inhibit adenylyl cyclase, thereby

potentially exerting negative inotropic effects (Hilal-dandan *et al*. 1994). The functional activity of Gi proteins maybe increased in SCI and antagonized the positive inotropic effects induced by Gq activation, this appears an likely explanation. An alternative possibility is that protein kinase C in SCI rat myocytes can not be activated by ET-1, which needs further study.

Studies on the effect of ET-1 on the voltage-dependent calcium current in cardiac myocytes under conventional whole cell configuration have yielded conflicting results with increase (Bkaily *et al*.1995), decrease (Bányász *et al*. 2001) as well as no effect (Habuchi *et al*.1992). These contradictory data may be caused by the wide differences in experimental conditions, including different species, tissues, preparations and tempertatures. In this study, we observed that exogenous ET-1 increased $I_{Ca,L}$ and had a positive inotropic effect in the control rat myocytes.However, ET-1 has no inotropic effects in SCI rat myocytes. Future studies are needed to investigate mechanisms underlying the absence inotropic effect of ET-1 in SCI rat myocytes.

## Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 30800375 and No.30670760) and grants from the Major State Basic Research Development Program of the People's Republic of China (No. 2006CB503807).

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**Fig.1.** Representative records of contractions (a and b) and the associated $Ca^{2+}$ transients (c and d) of control rat myocytes prior to (a and c) and after (b and d) application of ET-1 at 5 nmol/l for 10 min.

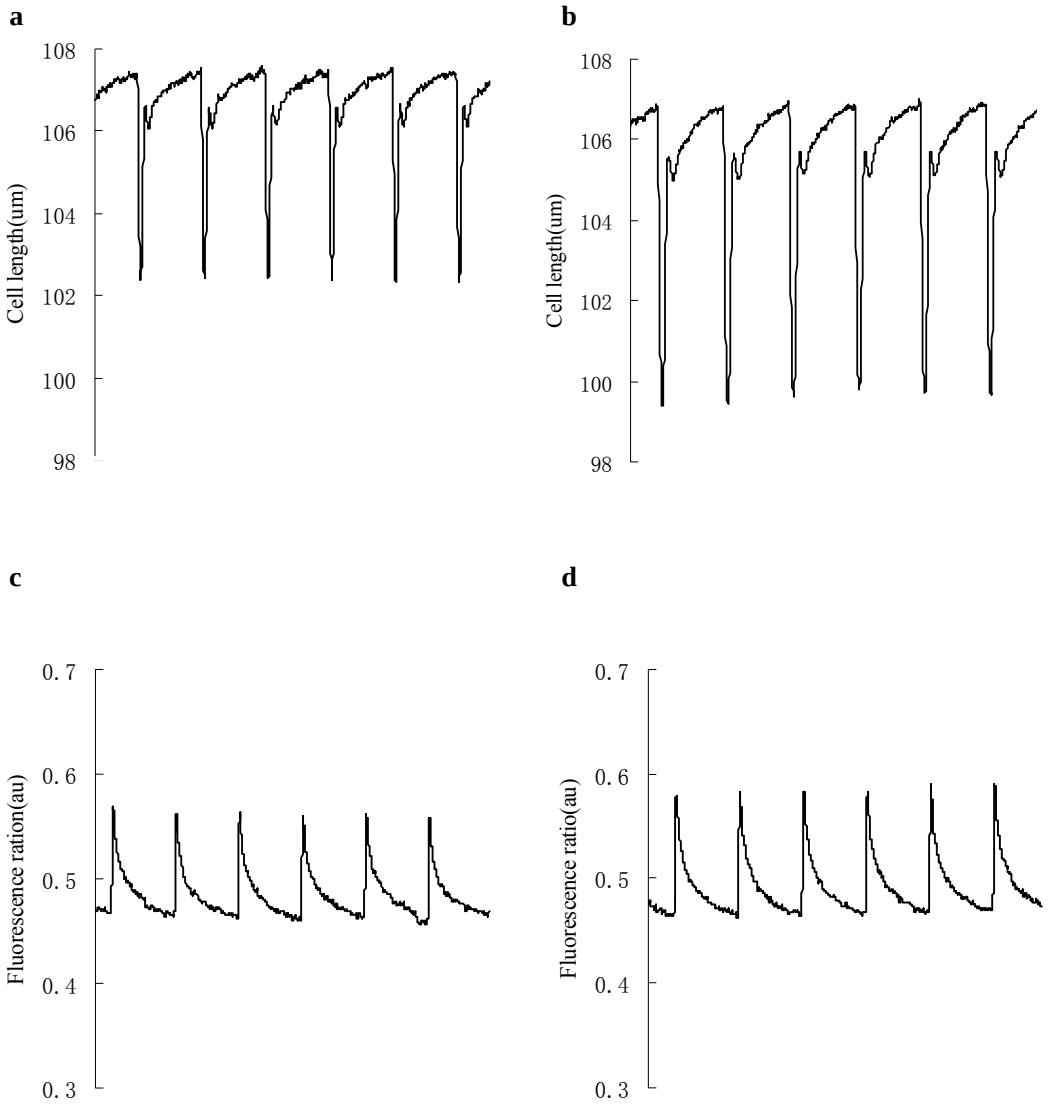


**Fig.2.** Representative records of contractions (a and b) and the associated $Ca^{2+}$ transients (c and d) of SCI rat myocytes prior to (a and c) and after (b and d) application of ET-1 at 5 nmol/l for 10 min.

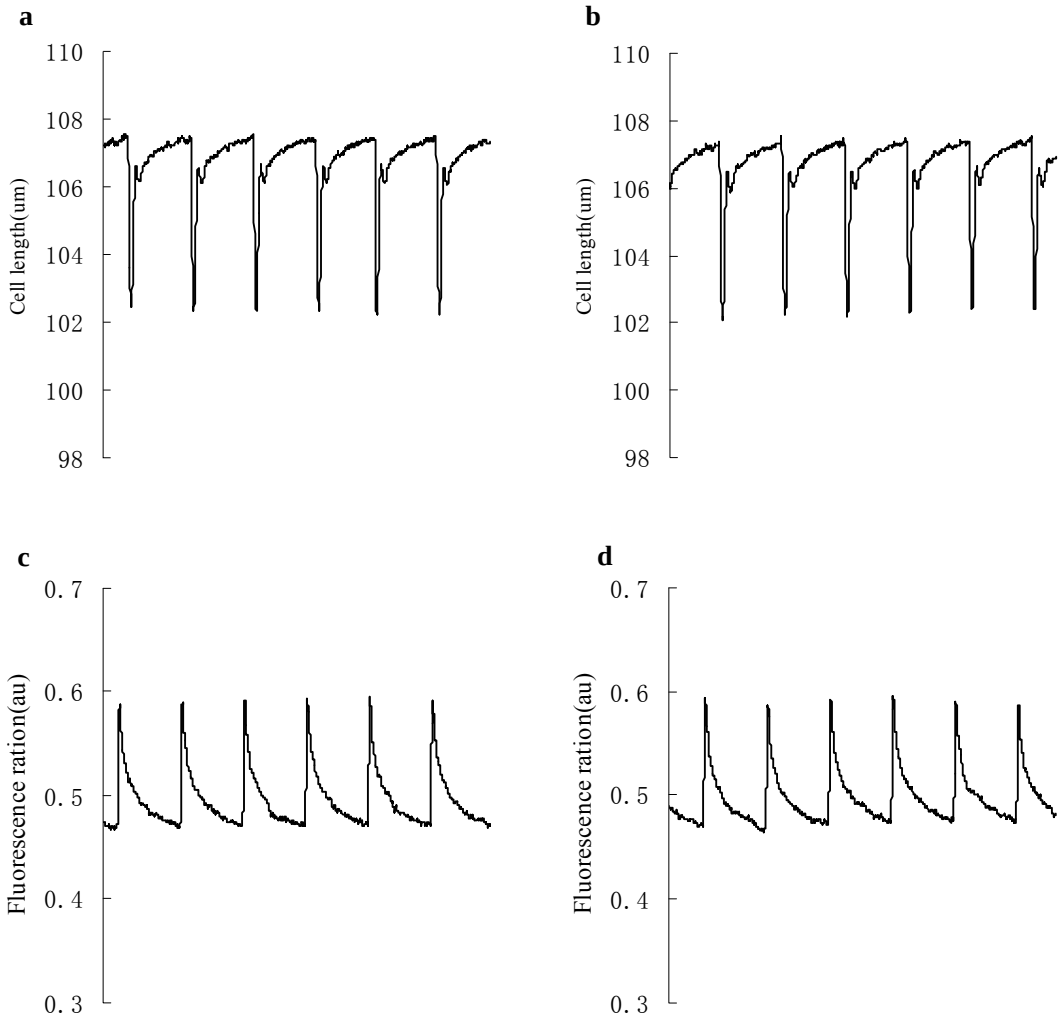
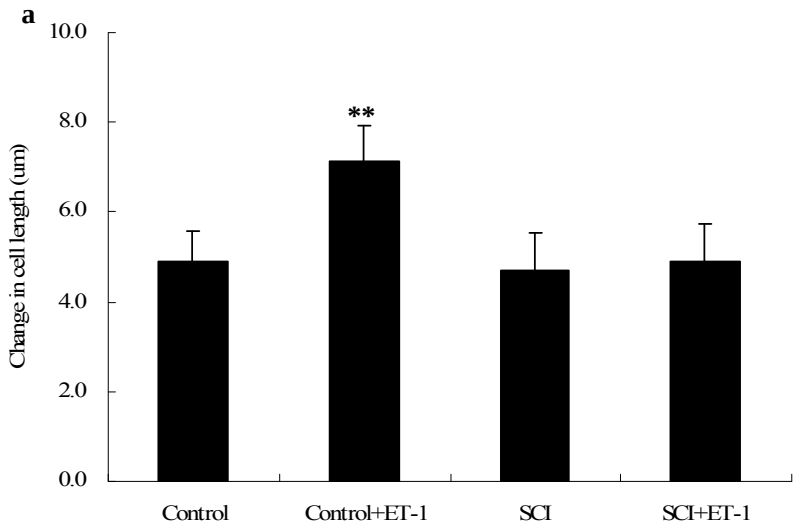
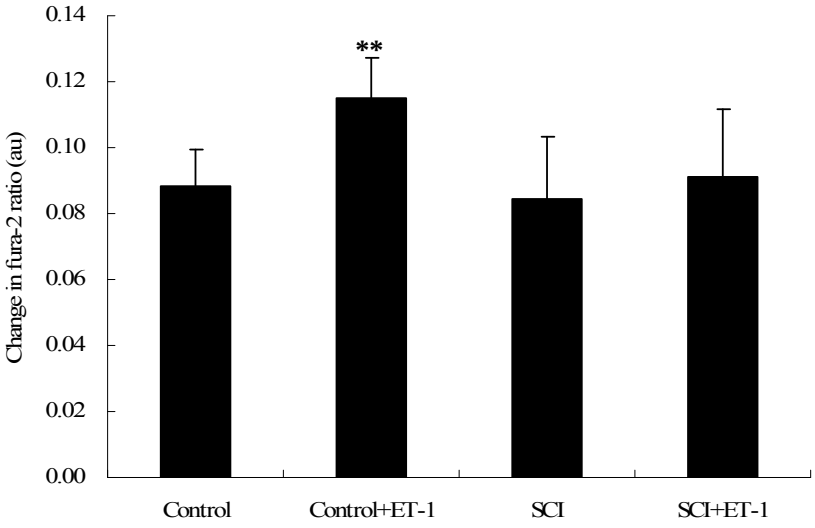


**Fig. 3.** (a) The mean values (±SD) of contraction amplitude in control and SCI group cells before and after application of ET-1 at 5 nmol/l are shown. (b) The mean values (±SD) are shown for $Ca^{2+}$ transient amplitude for control and SCI group cells before and after application of ET-1 at 5 nmol/l. Contraction amplitude and $Ca^{2+}$ transient amplitude were significantly increased after application of ET-1 at 5 nmol/l (n=12, **P<0.01) in control group cells, but not changed significantly in SCI group cells (n=12, P>0.05).



**b**


0.14
0.12
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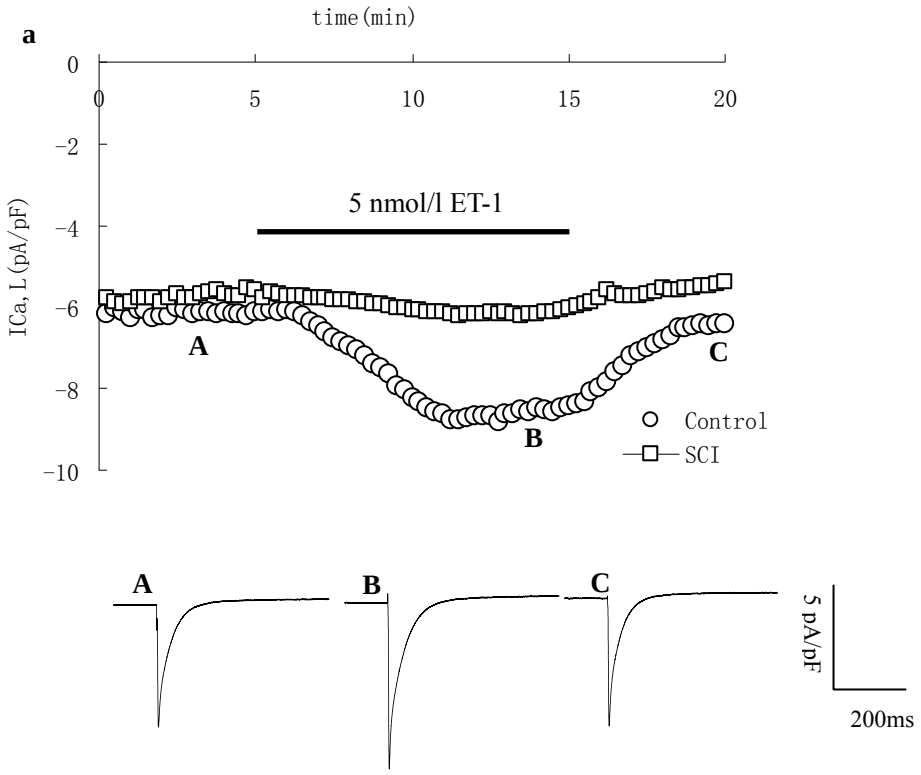
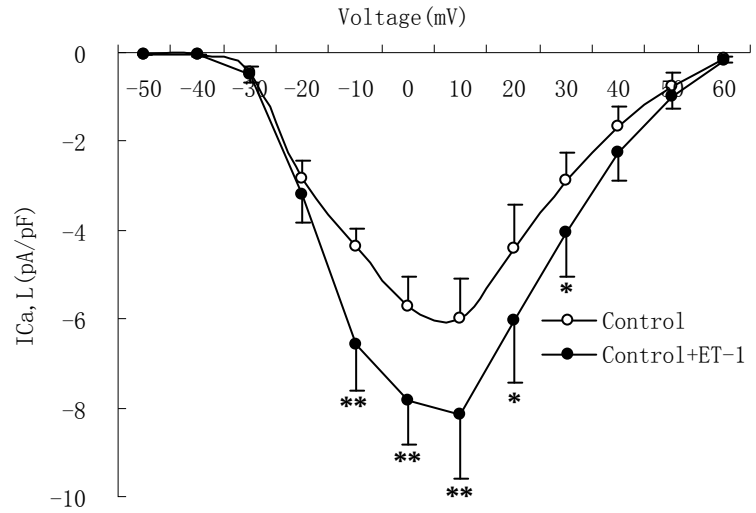
Change in fura-2 ratio (au)
**
Control
Control+ET-1
SCI
SCI+ET-1

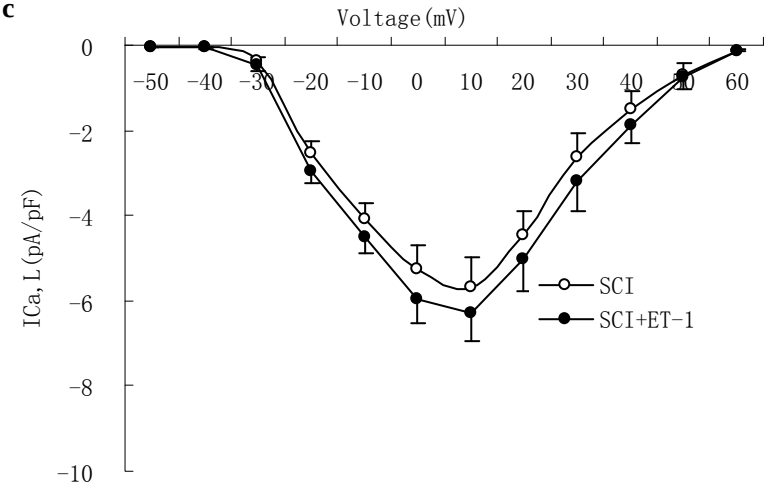
**Fig.4.** (a)A time course of the effect of 5 nmol/l ET-1 on peak $I_{Ca,L}$ density evoked by a step to +10 mV in a single myocyte of control rat (o)or SCI rat(□). Representative traces of $I_{Ca,L}$ of control myocyte corresponding to the points indicated at top are shown at bottom. (b,c) Effect of 5 nmol/l ET-1 on current-voltage(I-V) relationship curves of myocytes (n=20) of control rat (b)or SCI rat (c). I-V curves were obtained by plotting peak $I_{Ca,L}$ vs. test potential. Exposure to 5 nmol/l ET-1 resulted in a significant increase in peak $I_{Ca,L}$ of control rat myocytes but lack effect in peak $I_{Ca,L}$ of SCI rat myocytes. * $p<0.05$ vs control, ** $p<0.01$ vs control.



**b**


Voltage(mV)
0
−2
−4
−6
−8
−10
−50 −40 −30 −20 −10 0 10 20 30 40 50 60
ICa,L(pA/pF)
Control
Control+ET-1
**
**
**
*
*

**c**


Voltage(mV)
0
−2
−4
−6
−8
−10
−50 −40 −30 −20 −10 0 10 20 30 40 50 60
ICa,L(pA/pF)
SCI
SCI+ET-1