Reinduction of T-Type Calcium Channels by Endothelin-1 in Failing Hearts In Vivo and in Adult Rat Ventricular Myocytes In Vitro

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Background—In ventricular myocardium, the T-type Ca\(^2^+\) current (\(I_{\text{ca,T}}\)), which is temporarily observed during fetal and neonatal periods, has been shown to reappear in failing/remodeling hearts. However, its pathophysiological regulation has not been elucidated.

Methods and Results—We utilized Dahl salt-sensitive (DS) rats with hypertension at the stage of concentric left ventricular (LV) hypertrophy (11 weeks old, LVH) and at the heart failure stage (16 to 18 weeks old, CHF). Some were treated with bosentan (100 mg/kg per day) during the period from LVH to CHF. In LVH, neither the presence of \(I_{\text{ca,T}}\) (measured in the freshly isolated LV myocytes) nor an increase in \(\alpha\)-1G mRNA expression were detected. This condition was associated with increases in tissue angiotensin II (AII) but not with endothelin (ET)-1 peptides. In contrast, in CHF, when the tissue AII remained elevated and ET-1 de novo increased, \(I_{\text{ca,T}}\) was recorded in most of the cells (−0.87±0.18 pA/pF at −30 mV, \(P<0.01\) versus LVH). This was associated with a significant increase in the \(\alpha\)-1G mRNA level. The chronic bosentan treatment eliminated both the elevation of \(\alpha\)-1G mRNA level and \(I_{\text{ca,T}}\) from the cells, whereas it did not affect the cell size and membrane capacitance. In addition, 48-hour exposure to ET-1 but not AII induced \(I_{\text{ca,T}}\) in normal adult myocytes in culture from Sprague-Dawley rats.

Conclusions—\(I_{\text{ca,T}}\) channels reappear in failing but not in hypertrophied LV cardiomyocytes in a manner depending on the tissue ET-1 activation. (Circulation. 2003;108:2530-2535.)

Key Words: hypertension ■ heart failure ■ calcium ■ endothelin ■ angiotensin II

Despite the abundant expression of L-type Ca\(^2^+\) current (\(I_{\text{ca,L}}\)), T-type Ca\(^2^+\) current (\(I_{\text{ca,T}}\)) is scarcely observed in adult ventricular myocytes.\(^1\)\(^-\)\(^3\) Because of its transient expression during the embryonic/neonatal period,\(^4\) the major role of this type of Ca\(^2^+\) channel in myocardial tissue is assumed to be a mediation of Ca\(^2^+\) influx that promotes cell division and growth. Interestingly, recent studies indicate that \(I_{\text{ca,T}}\) also reappears in myocardium with left ventricular (LV) hypertrophy or cardiac failure and under certain neurohumoral stimulations.\(^5\)\(^-\)\(^9\) In these states, this transient channel may induce Ca\(^2^+\) overload, trigger-type arrhythmias, and Ca\(^2^+\)-dependent signaling that mediates cell apoptosis. Thus, inhibitors specific for this type of Ca\(^2^+\) channel may have clinical efficacy for preventing sudden death and progressive cardiac dysfunction in patients with diseased myocardium, although it has not yet been demonstrated.\(^10\)

We report that the reappearance of this type of Ca\(^2^+\) channel is tightly regulated by the local neurohumoral environment, especially by tissue endothelin (ET)-1 but not by angiotensin II (AII), through the use of cultured adult ventricular myocytes and an animal model in which the transition from compensatory LV hypertrophy to failure was distinctively observed.\(^11\)\(^-\)\(^13\) Our data suggest that suppression of \(I_{\text{ca,T}}\) may be achieved by pharmacological regulation over the tissue neurohumoral condition of the failing myocardium in this animal model.

Methods

Animal Model of Cardiac Hypertrophy and Failure

Male inbred Dahl salt-sensitive (DS) and salt-resistant (DR) rats fed an 8% NaCl (high-salt) diet, as described previously, were used.\(^11\)\(^-\)\(^13\) In DS rats, a state of compensatory concentric left ventricular hypertrophy (LVH, n=16) is obtained at the age of 11 weeks, followed by LV dilation, global hypokinesis, and pulmonary congestion (CHF, n=22) at the age of 16 to 18 weeks.\(^11\)\(^-\)\(^12\) Normotensive DR rats were used as age-matched control animals (11-week-old animals, n=16; 17-week-old, n=16). Fourteen LVH-DS rats were subjected to chronic treatment with bosentan (100 mg/kg per day

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ET-1 Induces $I_{Ca,T}$ in Ventricular Myocytes

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PO$^4$) until the age of 17 weeks to analyze the influence of ET-1 on $I_{Ca,T}$ expression. The Institutional Animal Care and Use Committee of Kyoto University Graduate School of Medicine approved the procedure.

Isolation and Primary Culture of Adult Rat Ventricular Myocytes

Single LV myocytes were obtained from DS and DR rats at the ages of 11 and 17 weeks (n=10 for each group), using enzymatic digestion as previously described.14

Adult ventricular myocytes were isolated from 11- to 13-week-old male Sprague-Dawley rats (n=7), purified (>98%), and primary-cultured in laminin-coated dishes, following the protocol used by Piper et al.5,16 and Fares et al.6 Myocytes were incubated under serum-free conditions for 48 hours at 37°C and then with either vehicle, 1×10^{-6} mol/L AII,17 1×10^{-7} mol/L ET-1,18 or 1×10^{-7} mol/L ET-1 containing 1×10^{-6} mol/L BQ23 for 48 hours.

Electrophysiological Recording

The ventricular myocytes from Dahl rats or primary-cultured adult myocytes from Sprague-Dawley rats were subjected to a patch-clamp study (Axopatch 200A with pClamp6.0, Axon Instruments Inc), using the whole-cell configuration at 37°C. After disrupting the seal, the external perfusate was switched from Tyrode solution to a tetrodotoxin (TTX)-containing Na$^+$-K$^+$-free solution. The $I_{Ca,T}$ and $I_{Ca,L}$ currents were discriminated by changing the holding potential ($V_h$) from -90 to -50 mV for 200 ms before applying 10-mV-stepped pulses (from -80 to 80 mV for 200 ms) for every 5-second interval. $I_{Ca,T}$ was obtained by subtracting these current sets at the different $V_h$ values. The voltage dependence of steady-state inactivation of the Ca$^{2+}$ currents was determined by using a double-pulse protocol in which conditioning pulses of 1-second duration to various voltages were followed by a 10-mV-test pulse for 300 ms. The voltage dependence for activation of Ca$^{2+}$ currents was also determined according to a method described by Ferrumi and Nathan.19 The activation parameter was estimated from the peak conductance by a Boltzmann distribution function.20

The normal Tyrode solution used contained the following (in mmol/L): NaCl 140, KCl 5.4, CaCl$_2$ 1.8, MgCl$_2$ 0.5, NaH$_2$PO$_4$ 0.33, glucose 5.5, and HEPES 5; the pH was adjusted to 7.4 with NaOH. The Na$^+$-K$^+$-free solution contained the following (in mmol/L): tetraethylammonium (TEA)-Cl$_n$, CaCl$_2$ 5.4, MgCl$_2$ 2, glucose 10, HEPES 10, and TTX 0.01; pH 7.4 with TEA-OH. The pipette solution contained the following (in mmol/L): CsCl 100, TEA-Cl 20, MgATP 5, NaGTP 0.2, EGTA 10, and HEPES 10; pH 7.3 with CsOH.

Competitive RT-PCR for Expression of $\alpha_2$G Ca$^{2+}$ Channel Subunit

Total RNA was isolated from LV tissue (n=6 for each group) by the acid guanidinium thiocyanate–phenol–chloroform method. After synthesis of the first-strand cDNA, a constant amount of cDNA was amplified by PCR with a serially diluted nonhomologous DNA fragment containing primer template sequences as a competitor (TaKaRa). Sense (S) and antisense (AS) primers for $\alpha_2$G (S, 5’-CACCCCAACAGTGGTACACC–3’ [positions 4238 to 4257], and AS, 5’-AGCCAGCTCAGTGCAGTA-3’ [4768 to 4787]; and for GAPDH (S, 5’-TTGCCATACAGGACCCCTCT–3’ [169 to 188], and AS, 5’-TTGCCATACAGGACCCCTCT–3’ [558 to 577]) were synthesized by using the published cDNA sequences.15,21 A portion of the PCR reaction product was then resolved by electrophoresis on a 1.5% agarose gel and photographed with Polaroid type 55 films. Quantitative evaluation was carried out with the use of scanning densitometric analysis.

Myocardial Content of ET-1 and All Peptides

All and ET-1 were extracted from LV tissue (n=6 for each group) according to the method previously described.15 All content was determined with a radioimmunoassay kit (Nichols Institute Diagnos-

Statistical Analysis

The results are expressed as mean values±SEM. One-way factorial ANOVA was used to compare parameters from different animal groups. Significant differences among groups (P<0.05) were detected by Fisher’s protected least significance test.

Results

$I_{Ca,T}$ was only detected in myocytes from CHF rats. Figure 1a shows representative membrane currents obtained in a hypertrophied LV myocyte from an LVH-DS rat. In this particular recording, the amplitude of Ca$^{2+}$ currents obtained at two distinct $V_{hp}$ levels appeared to be equivalent, thus indicating the absence of $I_{Ca,T}$. Figure 1b and 1c summarizes the current-voltage (I-V) relation of these two Ca$^{2+}$ currents obtained from LVH (n=23 cells) and age-matched DR myocytes (n=21). The data indicate that LVH per se was not a sufficient condition to induce $I_{Ca,T}$ in ventricular myocytes.

On the other hand, Figure 2a shows membrane currents obtained in a myocyte from a CHF-DS rat. Depolarizing pulses ($V_{hp}$, -90 mV) to -50 through -30 mV elicited inward currents that were absent when the $V_{hp}$ was switched to -50 mV, whereas depolarization to 0 mV showed equivalent inward currents at each $V_{hp}$ level. The subtraction currents between the two $V_{hp}$ levels were clearly inhibited by adding 50 μmol/L Ni$^{2+}$ to the external perfusate (Figure 2b), consistent with the characteristics of $I_{Ca,T}$. The I-V relations were summarized from 21 CHF-DS myocytes in which 17 of these showed subtraction currents (Figure 2c). The current was induced by depolarization above -60 mV, showed a peak at -30 mV (-0.9±0.2 pA/pF), and the reversal potential was +30 mV. The I-V relation relative to the $I_{Ca,L}$ showed no change when compared with those obtained in age-matched DR (n=21, Figure 2d) or LVH-DS myocytes, but it significantly decreased in amplitude (-7.5±0.6 pA/pF, at 0 mV). Figure 3 shows the steady-state activation and inactivation kinetics for $I_{Ca,T}$ and $I_{Ca,L}$ obtained in myocytes from CHF-DS rats (n=8 cells). The $I_{Ca,T}$ currents were activated during steps more positive than -60 mV and were fully activated at -10 mV; in contrast, the $I_{Ca,L}$ currents were activated at voltages more positive than -40 mV and were fully activated at 10 mV. The slope (k) and half-maximum voltage ($V_{0.5}$) of $I_{Ca,T}$ were 8.1±0.8 and -29.7±0.8 mV, respectively, for activation, and 5.5±0.1 and -51.5±0.1 mV for inactivation. In the same cells (n=8), the potential ranges for activation of $I_{Ca,L}$ were -40 to 30 mV (k=6.4±0.4 and $V_{0.5}$=-16.4±0.5 mV) and -40 to 0 mV for inactivation (k=5.4±0.1 and $V_{0.5}$=-31.5±1.4 mV). Taken together, the characteristics of the subtraction currents were all consistent with those known for $I_{Ca,T}$, and this current type was de novo detected in failing ventricular myocytes.

Figure 4 shows the semiquantitative levels of $\alpha_2$G mRNA in the LV myocardium from DS and DR rats (n=6 for each group). Compared with the age-matched DR, the LVH-DS myocardium showed a modest increase in the $\alpha_2$G mRNA (NS). Instead, it obviously increased in the CHF-DS myocardium. Thus, the transcription of the $\alpha_2$G gene might tightly regulate the functional property detected as $I_{Ca,T}$ in the failing heart.
Possible Induction of $I_{\text{Ca,T}}$ by Tissue ET-1

Our previous study showed a different time course of activation and pathological roles for AII versus ET-1 in the LV myocardium during heart failure transition in this particular animal model. As shown in Figure 5, consistent with the previous study, the tissue AII started to increase at the LVH stage and sustained the level then after. Instead, the ET-1 peptide in the same samples was inactive at the LVH stage and then showed de novo elevation at the CHF stage ($n=1000\pm6$ for each group). Interestingly, in ventricular myocytes ($n=16$) isolated from the bosentan-treated animals, whereas the membrane capacitance ($429\pm41\ pF$) was comparable to that of CHF cells ($435\pm30\ pF$), obvious $I_{\text{Ca,T}}$ currents were detected only in 2 cells ($P<0.05$, $\chi^2$ test). The reduction of peak $I_{\text{Ca,L}}$ observed in CHF myocytes was significantly restored ($10.3\pm0.8\ pA/pF$, at 0 mV) to the equivalent level in the age-matched control animals (Figure 2e). In addition, the increased $\alpha_1\text{G}$ mRNA level in the CHF myocardium returned to baseline after the bosentan treatment (Figure 4, $n=6$).

Induction of $I_{\text{Ca,T}}$ in In Vitro Adult Rat Ventricular Myocytes Exposed to ET-1 But Not to AII

The effect of ET-1 on $I_{\text{Ca,T}}$ was further examined in in vitro cultured adult myocytes. Figure 6a through 6c shows $I$-$V$ relations of $\text{Ca}^{2+}$ currents obtained in adult rat ventricular myocytes cultured with either vehicle, ET-1, or AII for 48 hours. $I_{\text{Ca,T}}$ was observed in myocytes (12 of 17 cells) with ET-1 but not in control (1 out of 21 cells) or with AII (3 of 16 cells). Figure 6d shows the relation in myocytes with ET-1 plus BQ123, an ETA receptor blocker. With BQ123, the $I_{\text{Ca,T}}$ was only detectable in 2 of 16 cells ($P<0.05$, $\chi^2$ test). $I_{\text{Ca,L}}$ was not affected in all groups.

Discussion

The present study demonstrated that the $I_{\text{Ca,T}}$ current reappears in ventricular myocytes isolated from hypertrophied and failing rat hearts in a manner parallel to the local ET-1 activation, and this reappearance is clearly inhibited by chronic ET-1 blockade. In addition, in vitro isolated adult rat ventricular myocytes showed that ET-1 stimulation induced $I_{\text{Ca,T}}$ in the absence of mechanical loading. Hence, the reap-
The reappearance of $I_{Ca,T}$ in adult ventricular myocytes might be regulated at least in part by local ET-1 activation that occurred during the heart failure transition in this animal.

$I_{Ca,T}$ and Cardiac Hypertrophy

Cardiac $I_{Ca,T}$ is generally observed in embryonic and neonatal ventricular myocytes but is known to disappear throughout postnatal development. In adulthood, $I_{Ca,T}$ is present exclusively in Purkinje cells, atrial myocytes, sinoatrial node cells, and sinus venosus cells but not in ventricular myocytes in rats. On the other hand, the reappearance of $I_{Ca,T}$ has been reported in ventricular myocytes isolated from aortic-banded rats and infarcted rats. These findings suggest that $I_{Ca,T}$ is related to cell growth, proliferation, and development and might be reintroduced by hemodynamic loading. To investigate the stage-specific expression of $I_{Ca,T}$ during the transition from LVH to CHF, we used DS rats with hypertension. In this animal, we demonstrated that $I_{Ca,T}$ did not appear in myocytes at the compensated LVH stage but did appear de novo in myocytes at the CHF stage. Our findings indicate that the cardiac hypertrophy induced by pressure overload is not necessarily accompanied by the reappearance of $I_{Ca,T}$, suggesting the possibility that factors other than the mechanical load might play roles in this reinduction of $I_{Ca,T}$.

Tissue ET-1 Activation Induces $I_{Ca,T}$

There are several lines of evidence that $I_{Ca,T}$ reappears due to certain neurohumoral stimuli. Xu et al reported that $I_{Ca,T}$ was
increased in atrial myocytes isolated from adult rats with growth hormone–secreting tumors, and Fares et al.⁸ reported that $I_{Ca,L}$ was observed in dedifferentiated adult ventricular myocytes cultured for 8 days with serum-containing media. Consistent with our previous reports,¹²,¹³ in DS rats at the LVH stage, the LV tissue ET-1 peptide level was within normal limits, contrasting with the abrupt and massive increase at the CHF stage. On the other hand, the tissue AII was already activated in the LVH stage and remained active throughout the study period.¹¹ It is generally assumed that both ET-1 and AII play hand-in-hand roles in promoting growth in the stressed myocardium²⁷; however, the difference in their time courses as well as the LV responses to their specific inhibitors¹³ inspired us to hypothesize that they may also participate in the induction of $I_{Ca,T}$ in a different manner.

Because of the time courses of these two active peptides and $I_{Ca,T}$ expression during the heart failure transition, we examined the chronic inhibition of the ET-1 system by bosentan. Consistent with our hypothesis, chronic bosentan therapy inhibited the transcription and induction of $I_{Ca,T}$ at the CHF stage.

### Figure 3
Voltage-dependent steady-state activation and inactivation of Ca²⁺ currents. Graph shows voltage dependence of the activation and inactivation of $I_{Ca,T}$ (●) and $I_{Ca,L}$ (□) in LV myocytes (n=8) isolated from failing DS rats. Continuous curves were obtained by fitting data to a Boltzmann distribution.

### Figure 4
Semi-quantitative RT-PCR data for measurement of α1G mRNA in the LV myocardium of DS and DR rats at the age of 11 weeks and 17 weeks. DS rats at the age of 17 weeks are subjected to chronic treatment with vehicle [Bos (−)] or with bosentan [Bos (+)]. Densitometric data are normalized by the corresponding amount of GAPDH (n=6 for each group).

### Figure 5
AII and ET-1 peptide levels in LV myocardium at two distinct stages at the age of 11 weeks (compensated LV hypertrophy) and 17 weeks (CHF) in DS rats (solid bars). Corresponding open bars indicate age-matched DR rats. n=6 for each group; *P<0.05.

### Figure 6
Average $I-V$ relationship of Ca²⁺ currents recorded in adult LV myocytes from Sprague-Dawley rats cultured with vehicle (a), $1 \times 10^{-7}$ mol/L ET-1 (b), or $1 \times 10^{-6}$ mol/L All (c) for 48 hours. Subtraction of Ca²⁺ currents (□) from HPs of −90 mV (□) and −50 mV (□) indicated that $I_{Ca,T}$ reappeared in LV myocytes exposed to ET-1 (b) but not to All (c). This ET-1–mediated $I_{Ca,T}$ reappearance was inhibited when myocytes were cultured with $1 \times 10^{-5}$ mol/L BQ123 (d).
The in vivo study left open the question of whether or not the inhibition of I_{Ca,T} reappearance by bosentan was the consequence of hemodynamic amelioration with stress reduction of the unit myocardium. Hence, we also performed current recordings using isolated adult ventricular myocytes that were serum-free cultured and then exposed to 1 × 10^{-6} mol/L AII or 1 × 10^{-7} mol/L ET-1 for 48 hours. The induction of I_{Ca,T} was observed after ET-1 stimulation but not after AII. This ET-1–mediated activation of I_{Ca,T} was totally blocked by the presence of BQ123, a specific ET_{A} receptor blocker. Taken together, our data confirmed that tissue activation of the ET-1 system during the heart failure transition might activate a reinduction of I_{Ca,T} through the ET_{A} receptor in a manner independent of mechanical loading. In the present study, we have not elucidated whether the promoter region of the α_{G} mRNA codes ET-1–regulated transcriptional factors such as GATA4 or whether the abundant activation of I_{Ca,T} during the embryonic stage also correlates with ET-1 activity. The abundant activation of ET-1 in the embryo and its critical roles in cell proliferation and differentiation may be consistent with this hypothesis; however, it needs further study.

**Detrimental Role of I_{Ca,T} in Diseased Myocardium**

The reappearance of I_{Ca,T} should induce changes in the electrophysiological properties of adult ventricular myocytes and may explain some aspects of the characteristics known in the failing myocardium. The activation/inactivation kinetics in failing myocytes showed that a “current window” for I_{Ca,T} exists between −60 and −20 mV, which might induce additional Ca^{2+} influxes. Although the mean I_{Ca,T} density recorded in these cells was 0.87 ± 0.18 pA/pF (with 5.4 nmol/L Ca^{2+}), the magnitude of which was much smaller (11%) than that of the peak I_{Ca,T}, the continuous influx of Ca^{2+} through the window at a membrane potential just above the normal resting level could lead to an intracellular Ca^{2+} overload and unexpected depolarizations during the diastolic period. Further studies are needed to elucidate the relations among the diastolic and systolic dysfunctions, Ca^{2+} regulation, and arrhythmogenesis of the failing myocardium relative to the I_{Ca,T} reappearance. However, the present study suggests that such known characteristics of the failing myocardium could be pharmacologically restored through the ET-1–mediated I_{Ca,T} reappearance.

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