Endothelin-1 (ET-1) is a 21-amino acid vasoconstrictive peptide originally derived from media bathing the primary cultures of porcine aortic endothelial cells. This endothelium-derived vasoconstrictive peptide induces a potent and sustained vasoconstrictive effect on a variety of blood vessels and may play an important role in the regulation of blood pressure and local blood flow. ET-1 has been shown to have specific receptors on the sarcolemma of cardiac myocytes, to increase the intracellular Ca²⁺ level ([Ca²⁺]i), and to act as a positive inotropic agent in mammalian atrial and ventricular muscle. Recently, we have reported that ET-1 induces hypertrophy with concomitant increases in the transcripts of muscle-specific genes and a proto-oncogene, c-fos, as well as DNA and protein synthesis in cultured neonatal rat ventricular myocytes.

The mechanism by which ET-1 induces a positive inotropic effect and hypertrophy in cardiac myocytes remains unknown. One possible mechanism is the rise in [Ca²⁺], induced by ET-1. The rise in [Ca²⁺], or induction of hypertrophy by ET-1 is not affected by L-type Ca²⁺ channel blockers nor caffeine or ryanodine, suggesting that these ET-1 actions result from the mobilization of calcium- and ryanodine-insensitive intracellular stores and/or from Ca²⁺ entry through the sarcolemma via a pathway that is not the voltage-dependent L-type Ca²⁺ channel. One possible pathway of Ca²⁺ entry through sarcolemma is the voltage-dependent T-type Ca²⁺ channel. Up to the present, however, no report has been made on the effect of ET-1 on T-type Ca²⁺ current (ICₜ). In the present experiments, we examined the effect of ET-1 on ICₜ and discussed the possible role of T-type Ca²⁺ channels on the ET-1-induced rise in [Ca²⁺], and hypertrophy in...
ventricular myocytes. Cultured neonatal rat ventricular myocytes were used in the present study because we had previously demonstrated hypertrophic action of ET-1 in the same experimental preparations.5

Materials and Methods

Cell Cultures

A primary culture of neonatal rat ventricular myocytes was prepared by the method originally described by Simpson and Savion,7 with minor modifications. Briefly, the hearts from 1- or 2-day-old Wistar rats (Japan Laboratory Animals, Tokyo) were minced and dissociated with 0.1% trypsin (Difco, Detroit, Mich.). After dispersed cells were incubated on 100-mm culture dishes (Falcon, Oxford, Calif.) for 60 minutes at 37°C in 5% CO2, nonattached viable cells were collected and seeded into 35-mm dishes. Ventricular myocytes were incubated in Eagle’s minimum essential medium (MEM) supplemented with 5% calf serum (Flow Laboratories, Inc., McLean, Va.) for 48 hours and then replaced with serum-free MEM for 48 hours. Several minutes before the experiments, the culture medium was replaced with tetrathylammonium (TEA) bath solution.

Solutions

TEA bath solution contained (mM) TEA-Cl 140, MgCl2 2, CaCl2 5.4, glucose 10, HEPES 10 (pH adjusted to 7.4 with TEA-OH), and tetrodotoxin (Sigma Chemical Co., St. Louis, Mo.) 0.01. When Ba2+ solution was used, 5.4 mM CaCl2 was simply replaced with 5.4 mM BaCl2. NiCl2 was prepared as 1 mM stock solution in distilled water. Nisoldipine, a gift of Bayer Japan, was prepared as 2 mM stock solution in dimethyl sulfoxide (DMSO). ET-1 (Peptide Institute, Osaka, Japan) was prepared as 0.1 mM stock solution in distilled water and stored at <−20°C until needed. Bovine serum albumin (BSA, Sigma) was added to the bath solution at a final concentration of 10 nM on the day of experiment. Phorbol 12,13-dibutyrate (PDbu, Sigma), 4β-phorbol 12-myristate 13-acetate (PMA, Sigma), and 4α-phorbol 12,13-dibutyrate (4α-PDbu, Sigma) were prepared as 1–2 mM stock solutions in DMSO and then stored at <−20°C until needed. These were added to the bath solution at a final concentration indicated in the text.

The internal solution for the suction pipette contained (mM) CsCl 100, TEA-Cl 20, EGTA 10, HEPES 10, ATP (as magnesium salt, Sigma) 5, and GTP (as lithium salt, Sigma) 0.2 (pH adjusted to 7.3 with CsOH). Stauroporine (Sigma) was prepared as 1 mM stock solution in DMSO and stored at 4°C. It was added to the pipette solution at a final concentration of 0.2 μM. Protein kinase C inhibitor 1-(5-isoxquinolinesulfonyl)-2-methylpipеразине (H-7, Seikagaku Kogyo Co., Tokyo) was prepared as 10 mM stock solution in distilled water and stored at 4°C. It was added to the pipette solution at a final concentration of 20 μM. The final concentration of DMSO contained in each solution was <0.05%.

Recording Methods and Data Analysis

Whole-cell membrane currents were recorded by the patch-clamp method described by Hamill et al8 using glass pipettes with a diameter of 1–2 μm. The resistance of the pipette was 5–8 MΩ when filled with the internal solution. The electrode was connected to the input stage of a patch-clamp amplifier (model Axopatch-1C, Axon Instruments, Inc., Burlingame, Calif.) with a feedback resistance of 100 MΩ. Electrical connection to the pipette and to the bath was made through a Ag/AgCl half-cell electrode. The electrode potential was adjusted to zero current between the pipette solution and the bath solution immediately before each cell was approached. The pipette capacitance was compensated by adjusting the fast capacity transient on the current tracing immediately before the cell membrane under the pipette pore was broken. In this condition, capacitive currents were obtained by the application of a 10-mV hyperpolarizing pulse, and the area under the capacity transient was integrated and used as a measure of whole-cell sarcolemmal surface, assuming a specific membrane capacity of 1 μF·cm−2.9 The mean sarcolemmal surface area calculated from 62 cells was 1,640±160 μm2. Thereafter, whole-cell capacitance and series resistance were compensated by minimizing the size and duration of whole-cell capacity transients.

Whole-cell membrane currents were elicited with voltage-step protocols described in the text. Electrical signals were digitized on-line by a 12-bit resolution Labmaster A/D converter (TecMar Scientific Solutions, Burlingame, Calif.) under the control of an IBM-AT computer and were stored on a hard disk. Data were analyzed using a software program (pCLAMP, version 5.5.1, Axon Instruments). Experiments were done at a room temperature of 22–24°C (for Figure 8, at 34–36°C).

All data are presented as mean±SD. Analysis of variance was used to examine the effects of ET-1, PDbu, or 4α-PDbu. If a significant F test was found, comparisons were made posteriori to determine differences in individual means at each test potential (Vh) using the Newman-Keuls test with software program MDFR (BMDP Statistical Software, Los Angeles, Calif.). Values of p<0.05 were considered significant.

Results

Ca2+ Channel Currents in Cultured Neonatal Ventricular Myocytes

Figure 1 depicts voltage-clamp experiments used to separate L-type Ca2+ current (ICa,L) and ICa,T. Membrane currents were elicited with 200-msec voltage steps applied every 3 seconds to various Vh's between −80 and +80 mV in 10-mV steps from two different levels of holding potentials (Vh's). The preliminary data in our preparations showed that, in the presence or absence of ET-1, ICa,T was fully activated at −90 mV and was almost completely inactivated at −50 mV, whereas ICa,L was still fully activated at −50 mV. Thus, voltage steps from −90 mV activated both ICa,L and ICa,T (total Ca2+ current ICa) (top tracings in Figure 1A), whereas voltage steps from −50 mV activated only ICa,T (middle tracings in Figure 1A). The subtraction of currents at a Vh of −50 mV from currents at a Vh of −90 mV resulted in ICa,T (bottom tracings in Figure 1A). The current amplitudes were measured by subtracting the current at the end of the voltage step from the initial inward peak. Figure 1B shows current density–voltage relations of total ICa (filled circles), ICa,L (open squares), and ICa,T (open reversed triangles) constructed from 11 cells. The acti-
vation threshold was -30 mV for \( I_{Ca,L} \) and -50 mV for \( I_{Ca,T} \). \( I_{Ca,L} \) reached the maximum value at a \( V_h \) of +10 mV. The maximum amplitude of \( I_{Ca,L} \) normalized to the cell membrane area was -9.7±2.3 \( \mu A/cm^2 \); \( I_{Ca,T} \) reached the maximum value at -20 mV. The maximum amplitude of \( I_{Ca,T} \) was -3.0±1.3 \( \mu A/cm^2 \).

We used the same voltage protocols to separate Ba\(^{2+} \) current through L- and T-type Ca\(^{2+} \) channels. As shown in Figure 2A, in the Ba\(^{2+} \) solutions, kinetics of currents through L-type Ca\(^{2+} \) channels changed drastically, resulting in large, slowly inactivating currents. Kinetics of currents through T-type Ca\(^{2+} \) channels did not significantly differ from those in the Ca\(^{2+} \) solution, showing a rapid inactivation. These data further confirm that both L- and T-type Ca\(^{2+} \) channels are present in ventricular myocytes of neonatal rats. Figure 2B shows current density-voltage relations of Ba\(^{2+} \) currents through total Ca\(^{2+} \) channels (filled circles), L-type Ca\(^{2+} \) channels (open squares), and T-type Ca\(^{2+} \) channels (open reversed triangles) obtained in six cells. These curves indicate that, compared with the Ca\(^{2+} \) currents, the maximum amplitude of Ba\(^{2+} \) currents through L-type channels increased and its voltage dependence shifted -20 mV in a negative direction, whereas the amplitude and voltage dependence of Ba\(^{2+} \) currents through T-type channels did not change. The activation threshold was -40 mV for \( I_{Ca,L} \) and -50 mV for \( I_{Ca,T} \). \( I_{Ca,L} \) reached the maximum value at a \( V_h \) of -10 mV. The maximum amplitude of \( I_{Ca,L} \) normalized to the cell membrane area was -12.4±4.3 \( \mu A/cm^2 \). \( I_{Ca,T} \) reached the maximum value at -20 mV. The maximum amplitude of \( I_{Ca,T} \) was -3.4±0.5 \( \mu A/cm^2 \).

To dissect the two currents by their pharmacological sensitivities, we applied nisoldipine to block \( I_{Ca,L} \) and Ni\(^{2+} \) to suppress \( I_{Ca,T} \). As shown in Figure 3, \( I_{Ca,T} \) represented as the subtracted currents at a \( V_h \) of -20 mV was not affected by 0.2 \( \mu M \) nisoldipine (bottom tracings in Figure 3), whereas currents elicited from a \( V_h \) of -50 mV (right tracings in Figure 3) were mostly eliminated in the presence of 0.2 \( \mu M \) nisoldipine. On the other hand, 100 \( \mu M \) Ni\(^{2+} \) eliminated the subtracted \( I_{Ca} \) without changing \( I_{Ca} \) from a \( V_h \) of -50 mV, suggesting its effect on \( I_{Ca,T} \) but not on \( I_{Ca,L} \) (Figure 4). These findings further confirmed that the \( I_{Ca} \) elicited from a \( V_h \) of -50 mV consisted mainly of \( I_{Ca,L} \), whereas the subtracted \( I_{Ca} \) consisted of \( I_{Ca,T} \).

**Effects of ET-1 on \( I_{Ca,T} \) and \( I_{Ca,L} \)**

To examine the effects of ET-1 on \( I_{Ca,T} \) and \( I_{Ca,L} \), ET-1 at a concentration of 10 nM was added to the bath solution after a 10-minute equilibration period. Voltage-clamp protocols were repeated at 5, 10, 15, and 20 minutes after the addition of ET-1. Thereafter, ET-1 was washed out, and voltage-clamp protocols were
Figure 2. Ba\(^{2+}\) current through T- and L-type Ca\(^{2+}\) channels. Panel A: A series of Ca\(^{2+}\) currents (I\(_{Ca}\)) were elicited from a holding potential (V\(_h\)) of -90 mV (top tracings) and -50 mV (middle tracings) to a test potential (V\(_t\)) of -30 mV (left tracings) and +10 mV (right tracings). Voltage protocol and current separation (Dif., bottom tracings) were the same as those described in Figure 1. Voltage steps to -30 mV only activated a rapidly inactivating current through the T-type channel, whereas voltage steps to +10 mV activated a large, slowly inactivating current through the L-type channel. Panel B: Peak current density–voltage relations were plotted for total I\(_{Ca}\) (closed circles), current through the L-type channel (I\(_{Ca,L}\), open squares), and current through the T-type channel (I\(_{Ca,T}\), open reversed triangles) obtained from six cells. Note that the threshold potential for Ba\(^{2+}\) current through the L-type channel and the membrane potential to reach its maximum shifted ~20 mV in a negative direction compared with the I\(_{Ca}\) (Figure 1B), whereas the voltage dependence of I\(_{Ca,T}\) did not differ from that of I\(_{Ca,L}\).

(A) Control

(B) Nisoldipine

Figure 3. Tracings showing the effects of nisoldipine on the T-type Ca\(^{2+}\) current (I\(_{Ca,T}\)) and the L-type Ca\(^{2+}\) current (I\(_{Ca,L}\)). The same voltage protocols as in Figures 1 and 2 were used to separate I\(_{Ca,T}\) and I\(_{Ca,L}\) in the control condition (panel A) and in the presence of nisoldipine (0.2 \(\mu\)M, panel B). Nisoldipine almost completely eliminated current elicited from a holding potential (V\(_h\)) of -50 mV to a test potential (V\(_t\)) of +10 mV (middle right tracing in panel B). On the other hand, nisoldipine was without effect on the subtracted current (Dif.) at a V\(_t\) of -30 mV (bottom left tracing in panel B).
shifted =10 mV in a negative direction. The maximum current density of $I_{Ca,T}$ in the control state was $-3.0\pm1.4$ $\mu$A/cm$^2$, that at 20 minutes after the addition of ET-1 was $-4.4\pm1.6$ $\mu$A/cm$^2$ ($p<0.01$ versus the control value), and that at 10 minutes after washout of ET-1 was $-3.1\pm1.4$ $\mu$A/cm$^2$ ($p=NS$ versus the control value).

We also studied the effect of ET-1 on $I_{Ca,L}$. Figure 6A shows representative tracings of $I_{Ca,L}$ at a $V_t$ of 10 mV in the control state, 20 minutes after the addition of ET-1 (10 nM), and 10 minutes after washout of ET-1. The mean current density–voltage relations of $I_{Ca,L}$ from 11 cells are shown in Figure 6B. The maximum current
density of $I_{\text{Ca,L}}$ in the control state was $-9.7 \pm 1.9 \mu A/cm^2$, that at 20 minutes after addition of ET-1 was $-6.3 \pm 1.7 \mu A/cm^2$ ($p<0.01$ versus the control value), and that at 10 minutes after washout of ET-1 was $-5.0 \pm 1.4 \mu A/cm^2$ ($p<0.01$ versus the control value).

It is well known that the amplitude of $I_{\text{Ca,L}}$ decreases spontaneously after establishment of the whole-cell clamp configuration ("run-down"), whereas the fluctuation pattern of the amplitude of $I_{\text{Ca,T}}$ is relatively unknown. Recently, Tseng and Boydjen reported that $I_{\text{Ca,T}}$ showed "run-up," which stabilized approximately 10 minutes after establishment of the whole-cell clamp configuration. To determine whether the increase in $I_{\text{Ca,T}}$ after the addition of ET-1 was due to the run-up phenomenon or to the specific effect of ET-1, we recorded $I_{\text{Ca,T}}$ without addition of ET-1 in five cells (left graph in Figure 7A). The current density of $I_{\text{Ca,T}}$ in the control state (at 10 minutes after establishment of the whole-cell configuration) and 20 minutes after the acquisition of control data is plotted against $V_n$. The current density of $I_{\text{Ca,T}}$ at each $V_n$ was almost identical between the control state and 20 minutes after the acquisition of control data. The voltages for the activation sequence or the maximum activation did not shift with time. Thus, neither the enhancement of $I_{\text{Ca,T}}$ nor the shift in its voltage dependence by ET-1 was due to spontaneous fluctuation after establishment of the whole-cell clamp configuration. It is also possible that the change in $I_{\text{Ca,T}}$ by addition of ET-1 was due to nonspecific effects of perfusion with peptides. To test this possibility, we recorded $I_{\text{Ca,T}}$ in the presence of denatured ET-1 by boiling at 95°C for 10 minutes in four cells (left graph in Figure 7B) and in the presence of BSA in four cells (left graph in Figure 7C). The current density of $I_{\text{Ca,T}}$ in the control state (at 10 minutes after establishment of the whole-cell configuration) and 20 minutes after the addition of heat-denatured ET-1 (10 nM) (left graph in Figure 7B) or BSA (20 nM) (left graph in Figure 7C) is plotted against $V_n$. In either case, the maximum current density of $I_{\text{Ca,T}}$ was not significantly changed from the control values, but the voltage for the activation sequence or the maximum activation shifted $\approx 10$ mV in the negative direction with time. These findings appear to suggest that the enhancement of $I_{\text{Ca,T}}$ by ET-1 was due to the specific effect of ET-1, whereas the shift in the voltage dependence of $I_{\text{Ca,T}}$ activation appears to be due to nonspecific effects of perfusion with peptides.

To test whether the specific action of ET-1 was responsible, at least in part, for the decrease in the amplitude of $I_{\text{Ca,L}}$, we also recorded $I_{\text{Ca,L}}$ for 20 minutes without the addition of ET-1 in five cells (right graph in Figure 7A), after addition of heat-denatured 10 nM ET-1 (right graph in Figure 7B), and after the addition of 10 nM BSA (right graph in Figure 7C). When ET-1 (10 nM) was applied to the bath solution, the amplitude of $I_{\text{Ca,L}}$ 20 minutes after ET-1 application was decreased to $52 \pm 19\%$ of the control value (see Figure 6B). When
ET-1 was not applied and only the time course of $I_{Ca,L}$ was monitored, the amplitude of $I_{Ca,L}$ 20 minutes after the acquisition of control data was 47±26% of the control values. The amplitude of $I_{Ca,L}$ 20 minutes after the application of heat-denatured ET-1 (10 nM) or BSA (10 nM) was 55±21% or 46±19% of the control values, respectively. These values are not significantly different from the values obtained when ET-1 (10 nM) was applied, suggesting only a minor effect of ET-1, if any, on $I_{Ca,L}$.

We also tested the effect of ET-1 on $I_{Ca,T}$ at a temperature of 34–36°C. Figure 8A displays the current density-voltage relations in the control state and 20 minutes after the addition of ET-1 (10 nM) (n=5). At a temperature of 34–36°C, the maximum amplitude of $I_{Ca,T}$ after the addition of ET-1 increased from the control value of $-3.2±0.8 \mu A/cm^2$ to $-4.3±0.6 \mu A/cm^2$ after the addition of ET-1 (p<0.01). Furthermore, we also tested the effect of ET-1 on Ba$^{2+}$ current through T-type channels (n=4, Figure 8B) and its effect on $I_{Ca,L}$ in the presence of 0.2 μM nisoldipine, a blocker of $I_{Ca,L}$ (n=4, Figure 8C). In the former case, the kinetics of Ba$^{2+}$ currents through T-type and L-type channels are distinctly different, making it easy to separate these two currents. In the latter case, $I_{Ca,L}$ was blocked by nisoldipine almost completely (see Figure 3); thus, $I_{Ca,T}$ could be obtained without interference by $I_{Ca,L}$. In both cases, the addition of ET-1 still enhanced the maximum current density through T-type Ca$^{2+}$ channels. The maximum Ba$^{2+}$ current density through T-type channels was $-3.4±0.5 \mu A/cm^2$ in the control state and $-4.5±0.7 \mu A/cm^2$ after the addition of 10 nM ET-1 (p<0.01). For $I_{Ca,T}$ in the presence of nisoldipine (0.2 μM), it was $-3.1±1.5 \mu A/cm^2$ in the control state and $-4.2±1.2 \mu A/cm^2$ after the addition of ET-1 (p<0.01).

To examine the relation between the concentration of ET-1 and the magnitude of enhancement of $I_{Ca,T}$ carried by Ca$^{2+}$, we tested the effect of ET-1 on the amplitude of $I_{Ca,T}$ at concentrations of 0.01, 0.1, 0.32, 1, 3.2, 10, and 100 nM (Figure 9). Representative tracings of $I_{Ca,T}$ at −30 mV before and after the addition of ET-1 at concentrations of 0.1, 1, and 10 nM are shown in Figure 9A. A concentration-response curve was constructed from 40 pooled data measurements (four to 11 measure-
ments for each point) (Figure 9B). The dose–response relation between the concentration of ET-1 and the percent increment in the $I_{Ca,T}$ amplitude was fitted to the Hill equation using a least-squares method with the assumption of the Hill coefficient being 1:

$$Y = A/[1 + (K_T/[ET-1])^n]$$  \hspace{1cm} (1)

where $A$ is the maximum response, $K_T$ is the $EC_{50}$ value, and $n$ is the Hill coefficient, which is assumed to be 1 in this case. The concentration of ET-1 that produced a half-maximal enhancement of $I_{Ca,T}$ ($EC_{50}$) was 1.26 nM. The enhancement of $I_{Ca,T}$ appeared to achieve its maximum response at a concentration of 10 nM, and the maximal percent increment of $I_{Ca,T}$ was 54%.

**Effects of ET-1 on the Decay Time Constant of $I_{Ca,T}$**

Next, we examined whether ET-1 had any effects on the time course of decay of $I_{Ca,T}$. The decay time course of $I_{Ca,T}$ could be fitted to a single exponential curve at any $V_T$. Thus, the decay time course of $I_{Ca,T}$ may be expressed as

$$I_{Ca,T} = -[A + B \cdot \exp(-t/\tau)]$$  \hspace{1cm} (2)

where $A$ is a constant and $B$ represents the extrapolated amplitude of each component at the onset of depolarization step ($t=0$). Time constants of decay of $I_{Ca,T}$ at $V_T$ values between −50 and 0 mV are plotted in Figure 10 ($n=11$). The time constant showed U-shaped voltage dependence, with a minimum value at −10 mV in the control state. The addition of ET-1 (10 nM) did not significantly change the time constant but shifted its voltage dependence to −10 mV in a negative direction. The minimum time constant of decay of $I_{Ca,T}$ was $11.2 \pm 2.1$ msec at −10 mV in the control condition and $12.5 \pm 1.0$ msec at −20 mV after the addition of ET-1 ($p=NS$).

**Effect of Protein Kinase C Inhibitors**

In vascular smooth muscle cells, the binding of ET-1 to its sarcolemmal receptor is known to increase the activity of intracellular protein kinase C through a pathway involving GTP-binding protein.14 In the follow-

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**FIGURE 8.** Effects of endothelin-1 (ET-1) on the T-type Ca$^{2+}$ current ($I_{Ca,T}$) at a temperature of 34–36°C (panel A), the Ba$^{2+}$ current through the T-type Ca$^{2+}$ channel (panel B), and $I_{Ca,T}$ in the presence of 0.2 μM nisoldipine (panel C). In each experimental condition, peak current density–voltage relations were plotted in the control condition and 20 minutes after the addition of ET-1 (10 nM). Note that ET-1 augments the peak amplitude of $I_{Ca,T}$ at a temperature of 34–36°C (panel A, $n=5$), with Ba$^{2+}$ as a charge carrier (panel B, $n=4$), or in the presence of 0.2 μM nisoldipine (panel C, $n=4$). *$p<0.01$ vs. control.

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**FIGURE 9.** Dose-dependent enhancement of the T-type Ca$^{2+}$ current ($I_{Ca,T}$) by endothelin-1 (ET-1). Panel A: A series of $I_{Ca,T}$s were elicited by voltage steps to a test potential of −30 mV before and after the addition of 0.1 nM ET-1 (top left tracing), 1 nM ET-1 (top right tracing), and 10 nM ET-1 (bottom left tracing), and two current tracings before and after the addition of ET-1 were superimposed. Panel B: Percent increases in $I_{Ca,T}$ were plotted against the logarithm of the concentration of ET-1. The graph is constructed from 40 pooled data measurements (four to 11 measurements for each point). The continuous line was calculated by fitting the Hill equation (Equation 1 in text) to the data.
ing series of experiments, we examined whether or not the activation of protein kinase C was also involved in the enhancement of $I_{Ca,T}$ by ET-1 in neonatal ventricular myocytes. In the first series of experiments, we studied the effect of protein kinase C inhibitor staurosporine (0.2 μM) on the enhancement of $I_{Ca,T}$ by ET-1. The mean current density–voltage relations of $I_{Ca,T}$ from four cells in the control condition and 20 minutes after addition of ET-1 (10 nM) in the experiments using pipette solution containing 0.2 μM staurosporine are plotted in Figure 11A. However, the increase in the peak amplitude of $I_{Ca,T}$ did not show a statistically significant change. The maximum current density of $I_{Ca,T}$ was −4.5±1.0 μA/cm² in the control condition and −4.7±1.3 μA/cm² after the addition of 10 nM ET-1 ($p=NS$).

We also tested the effect of protein kinase C inhibitor H-7 (20 μM) on the enhancement of $I_{Ca,T}$ by ET-1. The current density–voltage relations in the control state and 20 minutes after the addition of ET-1 (10 nM) using pipette solution containing 20 μM H-7 are plotted in Figure 11B (n=4). There was no increase in the peak amplitude of $I_{Ca,T}$; the maximum current density of $I_{Ca,T}$ was −3.4±0.9 μA/cm² in the control condition and −3.3±0.9 μA/cm² after the addition of 10 nM ET-1 ($p=NS$).

**Effects of Protein Kinase C Activation on $I_{Ca,T}$**

Since both inhibitors of protein kinase C that were used (staurosporine and H-7) are known to inhibit other classes of serine/threonine-type protein kinases, the elimination of ET-1 action by these agents is not sufficient to conclude that ET-1 action on $I_{Ca,T}$ was via a pathway involving the activation of protein kinase C. Thus, we next examined the effects of tumor-promoting phorbol esters that are known to cause the activation of protein kinase C both in vitro and in intact cells. The mean current density–voltage relations of $I_{Ca,T}$ from six cells in the control condition and 20 minutes after the addition of 0.2 μM PDBu are shown in Figure 12A. Similar to the effect of ET-1, the maximum amplitude of $I_{Ca,T}$ was increased from −4.2±0.5 μA/cm² in the control condition to −5.5±1.0 μA/cm² after the addition of 0.2 μM PDBu. The addition of another type of phorbol ester (PMA) also increased the peak amplitude of $I_{Ca,T}$ (n=3, data not shown).

Figure 12B displays the effect of the addition of PDBu (0.2 μM) on $I_{Ca,T}$ in the presence of 20 μM H-7 in the pipette solution (n=4). In the presence of 20 μM H-7, the addition of PDBu (0.2 μM) did not increase the maximum current density of $I_{Ca,T}$; the maximum current density of $I_{Ca,T}$ was −4.7±0.9 μA/cm² in the control condition and −4.4±0.9 μA/cm² after the addition of PDBu ($p=NS$). We also tested the effect of the 4α isomer of PDBu (4α-PDBu, 0.2 μM) on $I_{Ca,T}$ (Figure 12C, n=4), which is inactive in stimulating protein kinase C. The addition of 4α-PDBu did not increase the maximum current density of $I_{Ca,T}$; the maximum current density of $I_{Ca,T}$ was −4.1±0.9 μA/cm² in the control condition and −3.5±0.6 μA/cm² after the addition of 0.2 μM PDBu ($p=NS$). These findings further indicate that the effect of PDBu on $I_{Ca,T}$ enhancement was due to a specific effect of PDBu on stimulating protein kinase C rather than to its nonspecific effects.

**Effect of GTP on ET-1 Action**

As stated earlier, ET-1–induced vasoconstriction in vascular smooth muscle cells is known to act through increasing the activity of intracellular protein kinase C, and this action is mediated through a pathway involving the GTP-binding protein. In the present study, we have demonstrated that the enhancement of $I_{Ca,T}$ by ET-1 in neonatal ventricular myocytes may occur through stimulating the activity of protein kinase C.

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**Figure 11. Effects of staurosporine and protein kinase C inhibitor H7 on endothelin-1 (ET-1) action on the T-type Ca²⁺ current.** Currents were elicited in the control state and 20 minutes after the addition of 10 nM ET-1 with internal pipette solution containing 0.2 μM staurosporine (panel A, n=4) and 20 μM H7 (panel B, n=4). Peak current density–voltage relations were plotted in the control state (closed circles) and 20 minutes after the addition of ET-1 (open reversed triangles).
get some insight whether this stimulating action of protein kinase C also involves the GTP-binding protein in neonatal ventricular myocytes, we tested the effects of ET-1 on I_{Ca,L} in the absence of GTP in the pipette solution. Figure 13 displays the mean current density–voltage relations of I_{Ca,T} from four cells in the control condition and 20 minutes after the addition of ET-1 (10 nM), when GTP was omitted from the pipette solution. In the absence of GTP, ET-1 failed to enhance the amplitude of I_{Ca,T}; the maximum current density of I_{Ca,T} was $-2.7 \pm 0.6$ nA/cm$^2$ in the control condition and $-2.5 \pm 0.5$ nA/cm$^2$ 20 minutes after the addition of ET-1 ($p=\text{NS}$).

**Discussion**

The present study demonstrated for the first time that ET-1, a novel endothelium-derived vasoconstrictor peptide, enhanced Ca$^{2+}$ entry through voltage-dependent T-type Ca$^{2+}$ channels in neonatal cultured rat ventricular myocytes. This ET-1 action appears to act through a pathway involving the activation of protein kinase C.

Although both types of I_{Ca}, high-threshold I_{Ca} (I_{Ca,L}) and low-threshold I_{Ca} (I_{Ca,T}), have been demonstrated in most cardiac cells,9,10,11 the physiological role of I_{Ca,T} in cardiac myocytes is largely unknown, except for its contribution to pacemaker potentials in sinoatrial node cells.20 In adult ventricular myocytes, the density of I_{Ca,T} seems sparse, and its presence has been reported mostly in cells from the guinea pig.20,21 In contrast, embryonic cardiac tissues display robust I_{Ca,Ts} that are apparently lost as the tissues mature.22 Kawano and DeHaan22 found that, in the ventricular myocytes from 14-day embryonic chick heart cells, the density of I_{Ca,T} was roughly twice as large as that of I_{Ca,L}. In the present article, we have confirmed the existence of robust I_{Ca,Ts} and I_{Ca,L} in rat neonatal ventricular myocytes. In ventricular myocytes from adult rat hearts, no measurable I_{Ca,Ls} have been reported. In neonatal rats, we found that the density of I_{Ca,T} was roughly one quarter that of I_{Ca,L}. In neonatal rat ventricular cells, the characteristics of I_{Ca,L} and I_{Ca,T} resembled those in other preaparations.9,10,11 Compared with I_{Ca,L}, the threshold potential for I_{Ca,T} activation and the membrane potential to reach its maximum were lower than those for I_{Ca,L}. Although we did not quantitatively compare decay time constants of the two Ca$^{2+}$ currents, the decay time course of I_{Ca,T} seemed to be faster than that of I_{Ca,L}. This difference in kinetics became more prominent when Ba$^{2+}$ was used as a charge carrier. Ba$^{2+}$ current through the L-type Ca$^{2+}$ channel inactives very slowly, whereas Ba$^{2+}$ current through the T-type Ca$^{2+}$ channel shows a rapid inactivation (see Figure 2A). Pharmacological features of I_{Ca,L} and I_{Ca,T} in neonatal rat ventricular myocytes were also similar to those in other preparations.9,10,11 I_{Ca,T} was diminished by 100 $\mu$M Ni$^{2+}$ without showing sensitivity to low concentrations of nisoldipine (0.2 $\mu$M) (see Figures 3 and 4).

The data in the present study show that ET-1 enhances the amplitude of I_{Ca,T}, whereas ET-1 appears to have no effect on its decay time course. One might argue

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**Figure 12.** Effect of activation of protein kinase C on the T-type Ca$^{2+}$ current (I_{Ca,T}) H7, protein kinase C inhibitor; PDBu, phorbol 12,13-dibutyrate. Panel A: Peak current density–voltage relations of I_{Ca,T} from six cells were plotted in the control condition (closed circles) and 20 minutes after the addition of 0.2 $\mu$M PDBu (open reversed triangles). $^a$p<0.05 and $^b$p<0.01 vs. control. Panel B: Peak current density–voltage relations of I_{Ca,T} were plotted in the control condition (closed circles) and 20 minutes after the addition of 0.2 $\mu$M PDBu (open reversed triangles) with 20 $\mu$M H7 in the pipette solution (n=4). Panel C: Peak current density–voltage relations of I_{Ca,T} were plotted in the control condition (open circles) and 20 minutes after the addition of a 4$\alpha$ isomer of PDBu (4$\alpha$-PDBu, 0.2 $\mu$M; open reversed triangles) (n=4).

**Figure 13.** Effects of endothelin-1 (ET-1) on the T-type Ca$^{2+}$ current (I_{Ca,T}) in the absence of GTP in the pipette solution. Peak current density–voltage relations of I_{Ca,T} in the control state and 20 minutes after the addition of 10 nM ET-1 were plotted for four cells in the absence of GTP in the pipette solution.
that the enhancement after addition of ET-1 was erroneously obtained because of the incomplete isolation of ICa,T using voltage clamp. To exclude this possibility, we also studied the effect of ET-1 on Ba2+ current or ICa,T in the presence of the L-type Ca2+ channel blocker nisoldipine. Ba2+ currents through T-type and L-type channels have distinctly different kinetics, making it easy to differentiate between the two currents. In our preparations, 0.2 μM nisoldipine almost completely suppressed ICa,L. Thus, in the presence of nisoldipine, ICa,T consists mostly of ICa,T. In both experimental conditions, ET-1 still augmented currents through T-type Ca2+ channels. Analysis of the effect of ET-1 on ICa,T was hampered by the run-down phenomenon. Nevertheless, the decrease in ICa,T upon perfusion of ET-1 was not significantly different from that in the absence of ET-1 or during the perfusion of denatured ET-1 or BSA, suggesting that ET-1 does not enhance the amplitude of ICa,T significantly. This finding appears to be in agreement with the data by Vigne et al., who demonstrated that the rise of [Ca2+]i in neonatal rat atrial cells induced by ET-1 was not blocked by L-type Ca2+ channel blocker, (+)-verapamil, or (+)-PN200-110. The effect of ET-1 on ICa,T was dose dependent. The enhancement of ICa,T by ET-1 was initiated at relatively low doses (0.32 nM), and the maximal response was attained at approximately 10 nM. The dose of ET-1 that induced half-maximal enhancement of ICa,T was approximately 1.26 nM. These data show close similarity to the dose–response relation for the rise in [Ca2+]i by ET-1 in cultured neonatal rat atria.4 The ET-1–induced rise of [Ca2+]i was initiated at a dose >0.3 nM and attained the maximal response at 10 nM. Furthermore, the dose–response relation for the induction of hypertrophy by ET-1 in neonatal cultured rat ventricular myocytes appeared to be similar.5 Effects of ET-1 on muscle-specific gene expression and on the synthesis of protein and DNA were initiated at a dose >0.1 nM and attained the maximal effect at a concentration of 10 nM.5

Augmentation of ICa,T induced by ET-1 appears to act through the activation of protein kinase C. Inclusion of staurosporine or H-7 in the intracellular solution incompletely, but to a substantial extent, antagonized the action of ET-1 on ICa,T. At this concentration, staurosporine is reported to inhibit the action of several classes of serine/threonine protein kinases, including protein kinase C, cAMP-dependent protein kinase, cGMP-dependent protein kinase, and Ca2+/calmodulin–dependent protein kinase II.17 H-7 at this concentration also inhibits both protein kinase C and cAMP-dependent protein kinase.16 Thus, antagonistic action of these agents may not be sufficient to conclude that protein kinase C is involved. Stimulation by tumor-promoting phorbol esters PDBu and PMA enhanced the peak amplitude of ICa,T. Although we have not directly measured protein kinase C activity, it is generally agreed that phorbol esters, like diacylglycerol, bind to the protein kinase C–phospholipid complex to cause stimulation of the enzyme.18 The stimulating effect by phorbol esters was prevented when H-7 was present in the pipette solution. Furthermore, the 4α isomer of PDBu (4α-PDBu), an inactive form of phorbol ester,19 failed to enhance ICa,T. Therefore, we speculate that protein kinase C activation mediates the enhancement of ICa,T induced by ET-1. The effects of the activation of protein kinase C on both ICa,L and ICa,T are currently controversial. Leatherman et al.23 have reported that phorbol esters reduce 42Ca uptake and [Ca2+]i, in cultured chick heart cells, implicating depressant effects of phorbol esters on sarcolemmal Ca2+ currents, whereas Lasheva et al.24 have reported augmentation followed by inhibition of ICa,L by phorbol esters in neonatal rat ventricular myocytes. Marchetti and Brown25 have shown that phorbol esters decrease both ICa,L and ICa,T in GH3 cells. Only minimal data are available, however, on the effect of phorbol esters on cardiac ICa,T. Recently, Tseng and Boyden26 reported a depressant effect of phorbol esters on ICa,T at a temperature of 36°C. To determine whether different experimental temperatures resulted in the different findings of the effect of phorbol esters on ICa,T in their study and ours, we performed experiments at a temperature of 34–36°C, resulting in the augmentation of ICa,T even at this temperature. Tseng and Boyden26 used ventricular and Purkinje cells freshly isolated from adult dogs; we used neonatal rat ventricular cells cultured for 4 days. Thus, the different findings may be due to an age-dependent action of protein kinase C, species difference, or altered properties after cell culture. It is also possible that the different findings were due to the different pipette solutions used.

Identification and pursuit of growth signals that trigger the expression of proto-oncogenes, which in turn direct the characteristic increase in protein synthesis, have been sought for years and remain to be settled. There is a compelling body of data suggesting that the increase in [Ca2+]i constitutes the crucial link between the initial stimulus for hypertrophy, such as elevated perfusion pressure and growth hormone, and the alterations in gene expression (see the review by Marban and Koretsune27). Xu and Best28 recently have demonstrated a threefold increase in the density of ICa,T in atrial myocytes obtained from young adult rats bearing growth hormone–secreting tumors. Therefore, it seems plausible that the enhancement of ICa,T may play a key role in the genesis of cardiac hypertrophy at least in the model in which hypertrophy is induced by stimulation of the sarcolemmal receptor of cardiac myocytes, although a further investigation is necessary to specify its exact role in hypertrophy.

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Endothelin-1 enhances calcium entry through T-type calcium channels in cultured neonatal rat ventricular myocytes.

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