Angiotensin II Signaling Pathways Mediate Expression of Cardiac T-Type Calcium Channels

Laurent Ferron, Véronique Capuan, Yann Ruchon, Edith Deroubaix, Alain Coulombe, Jean-François Renaud

Abstract—Recent studies indicate that cardiac T-type Ca\textsuperscript{2+} current (I\textsubscript{CaT}) reappears in hypertrophied ventricular cells. The aim of this study was to investigate the role of angiotensin II (Ang II), a major inducer of cardiac hypertrophy, in the reexpression of T-type channel in left ventricular hypertrophied myocytes. We induced cardiac hypertrophy in rats by abdominal aorta stenosis for 12 weeks and thereafter animals were treated for 2 weeks with losartan (12 mg/kg per day), an antagonist of type 1 Ang II receptors (AT\textsubscript{1}). In hypertrophied myocytes, we showed that the reexpressed I\textsubscript{CaT} is generated by the Ca\textsubscript{V3.1} and Ca\textsubscript{V3.2} subunits. After losartan treatment, I\textsubscript{CaT} density decreased from 0.40±0.05 pA/pF (n=26) to 0.20±0.03 pA/pF (n=27, P<0.01), affecting Ca\textsubscript{V3.1}- and Ca\textsubscript{V3.2}-related currents. The amount of Ca\textsubscript{V3.1} mRNA increased during hypertrophy and retrieved its nonhypertrophic level after losartan treatment, whereas the amount of Ca\textsubscript{V3.2} mRNA was unaffected by stenosis. In cultured newborn ventricular cells, chronic Ang II application (0.1 μmol/L) also increased I\textsubscript{CaT} density and Ca\textsubscript{V3.1} mRNA amount. UO126, a mitogen-activated protein kinase kinase-1/2 (MEK1/2) inhibitor, reduced Ang II–increased I\textsubscript{CaT} density and Ca\textsubscript{V3.1} mRNA amount. Bosentan, an endothelin (ET) receptor antagonist, reduced Ang II–increased I\textsubscript{CaT} density without affecting the amount of Ca\textsubscript{V3.1} mRNA. Finally, cotreatment with bosentan and UO126 abolished the Ang II–increased I\textsubscript{CaT} density. Our results show that AT\textsubscript{1}-activated MEK pathway and autocrine ET-activated independent MEK pathway upregulate T-type channel expression. Ang II–increased I\textsubscript{CaT} density observed in hypertrophied myocytes may play a role in the pathogenesis of Ca\textsuperscript{2+} overload and arrhythmias seen in cardiac pathology. (Circ Res. 2003;93:1241-1248.)

Key Words: angiotensin II  mitogen-activated protein kinase  T-type Ca\textsuperscript{2+} channel  cardiac hypertrophy  gene expression

The electrophysiological remodeling that occurs during cardiac pathologies, such as hypertrophy, contributes to myocardial remodeling and plays an important role in the development of arrhythmia, increasing the risk of morbidity and mortality.\textsuperscript{1} Reappearance of the T-type Ca\textsuperscript{2+} current (I\textsubscript{CaT}) has been observed in hypertrophied ventricular cells, in association with pressure overload, postinfarction, and cardiomyopathy.\textsuperscript{2-4} Little is known about the pathophysiological role of I\textsubscript{CaT}, but its activation at low voltage suggests that I\textsubscript{CaT} may be related to Ca\textsuperscript{2+} overload and arrhythmias.\textsuperscript{4} The relationship between I\textsubscript{CaT} and cardiac damage is highlighted by the therapeutic benefits of blocking I\textsubscript{CaT}.\textsuperscript{5,6} For example, the use of a selective I\textsubscript{CaT} antagonist, mibefradil, has been shown to improve survival in a rat model of chronic heart failure.\textsuperscript{7}

Previous studies showed that I\textsubscript{CaT} is developmentally regulated in rodent ventricular cells.\textsuperscript{8,9} In the fetus, I\textsubscript{CaT} is generated by both Ca\textsubscript{V3.1} (α1G) and Ca\textsubscript{V3.2} (α1H) pore-forming channels whereas in newborn rat ventricles, only Ca\textsubscript{V3.1}-related current can be recorded. Furthermore, I\textsubscript{CaT} is no more detectable in adult rat ventricles. The cardiac pattern of expression of T-type channels, together with previous reports describing the relationship between I\textsubscript{CaT} and cell proliferation, is consistent with Ca\textsuperscript{2+} uptake through I\textsubscript{CaT} being dedicated to specific functions related to the cell cycle.\textsuperscript{10-12} I\textsubscript{CaT} density increases after growth hormone stimulation,\textsuperscript{13} and thus I\textsubscript{CaT} may also be associated with growth processes in nonproliferating adult hypertrophied myocytes.

Few studies have been reported concerning the effectors/mechanisms involved in regulation of the expression of cardiac T-type channels.\textsuperscript{14} Among the factors stimulated during cardiac hypertrophy, it has been shown that acute application of endothelin (ET)-1 to cultured newborn rat ventricular cells increases I\textsubscript{CaT} density due to the activation of protein kinase C.\textsuperscript{15} Conflicting results have been obtained concerning adrenergic stimulation, which has been reported to have no effect or to increase the density of I\textsubscript{CaT} in the ventricle.\textsuperscript{16,17} To our knowledge, the only study describing the effects of angiotensin II (Ang II) stimulation in cardiac tissue was performed in frog atrial cells and reported an

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increase in $I_{\text{CaT}}$ density in response to acute Ang II application.  

In the heart, Ang II is known to play a critical role in ventricular hypertrophy and several aspects of signal transduction in response to Ang II stimulation resemble features of the signal transduction induced by growth factors. Ang II exerts its hypertrophic effect by activating G-protein-coupled type 1 Ang II receptors (AT$_1$) and initiating a number of well-defined intracellular signaling pathways. Considerable efforts are currently being made to identify the signaling molecules involved in hypertrophic responses, such as those involved in the mitogen-activated protein kinase (MAPK) cascade. Recent data have demonstrated that a MAPK cascade component, the extracellular signal-regulated kinase 1/2 (ERK1/2) activated by the MAPK kinase-1/2 (MEK1/2), may be an important component connecting several signaling pathways involved in hypertrophy.

In this study, we demonstrate for the first time that Ca$^{2+}$ entry through $I_{\text{CaT}}$ is increased by the Ang II–activated AT$_1$ cascade in left ventricular hypertrophied myocytes. We also show that Ca$_{\text{b}}$3.1 and Ca$_{\text{b}}$3.2 subunits participated to the reexpressed $I_{\text{CaT}}$ and these subunits are differently regulated. Finally, Ang II–activated MEK pathway and ET-activated independent MEK pathway are shown to regulate the expression of $I_{\text{CaT}}$-related subunits.

### Materials and Methods

#### Hypertrophic Model

Animals were cared for and used in accordance with the European convention for the protection of vertebrate animals used for experimental purposes, and institutional guidelines n° 86/609/CEE November 24, 1986. Adult male Wistar rats (IFFA Credo, France) weighing 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Stenosed rats (AS) were paired 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Stenosed rats (AS) were paired 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Adult male Wistar rats (IFFA Credo, France) weighing 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Stenosed rats (AS) were paired 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Adult male Wistar rats (IFFA Credo, France) weighing 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Stenosed rats (AS) were paired 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Adult male Wistar rats (IFFA Credo, France) weighing 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Stenosed rats (AS) were paired 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Adult male Wistar rats (IFFA Credo, France) weighing 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Stenosed rats (AS) were paired 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Adult male Wistar rats (IFFA Credo, France) weighing 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Stenosed rats (AS) were paired 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Adult male Wistar rats (IFFA Credo, France) weighing 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Stenosed rats (AS) were paired 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Adult male Wistar rats (IFFA Credo, France) weighing 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described. Stenosed rats (AS) were paired 150 to 170 g were subjected to abdominal aortic stenosis during 6 or 12 weeks as previously described.

#### Isolation of Adult Cardiomyocytes and Culture of Newborn Cardiomyocytes

Left ventricular myocytes were isolated enzymatically from adult rat hearts by retrograde perfusion, as described elsewhere. Cultures of newborn myocytes were prepared from the ventricles of 1- to 2-day-old Wistar rats, as described by Renaud et al. Twenty-four hours after plating, myocytes were incubated for 48 hours with Ang II (0.1 μmol/L, Sigma) or Ang II plus antagonists/inhibitors in fetal calf serum-free medium supplemented with 10% heat-inactivated horse serum. The antagonists were AT receptor antagonists (losartan, 1 μmol/L and PD123319, 10 μmol/L; Sigma) and ET receptor antagonists (bosentan, 0.1 μmol/L, generously provided by Actelion, BQ123, 10 μmol/L, and BQ788, 10 μmol/L; Sigma). For MEK1/2 inhibition, we used U0126, 10 μmol/L, and PD98059, 10 μmol/L (Cell Signaling Technology).

### Electrophysiological Recordings

Ca$^{2+}$ currents were recorded by the whole-cell patch-clamp technique at room temperature (22°C to 24°C). The fire-polished pipettes used had a resistance of 1 to 3 MΩ when filled with pipette solution (in mmol/L: CsCl 10, Cs-aspartate 120, MgCl$_2$ 3, HEPES 10, EGTA 15, CaCl$_2$ 1.8, glucose 10, creatine phosphate 3.6, MgATP 5, Tris-GTP 0.2, pH 7.2 adjusted with CsOH). Ca$^{2+}$ currents were recorded in an external solution containing 135 mmol/L TEA Cl, 1 mmol/L MgCl$_2$, 10 mmol/L CaCl$_2$, 10 mmol/L HEPES, 10 mmol/L glucose, 3 mmol/L 4-aminopyridine, 20 mmol/L CsCl, 0.03 mmol/L tetrodotoxin, pH 7.4 adjusted with CsOH. Additional details of recording and data analysis can be found elsewhere.

### Quantitative RT-PCR

Total RNA was prepared from ventricular tissues using Trizol (GIBCO-BRL). Reverse transcription (RT) and polymerase chain reaction (PCR) conditions, and the procedures used for quantification have been described elsewhere. Briefly, quantitative RT-PCR was performed with normalized RNA aliquots (1 μg) and a known amount of cRNA internal control (0.3×10$^6$ molecules for both transcript species, for tissues, and 2.5×10$^6$ molecules for cultures). The internal control differed from the target counterpart by only one restriction site. PCR was performed with a trace amount of 32P-labeled 5' primer. The number of copies per μg of total RNA was calculated using the following equation: [Internal control weight/ (size in nucleotides×330)]×6.02×10$^3$, and from the relationship [molecules cRNA control/cpm cRNA control]×cpm target=target molecules per normalized RNA aliquot.

### Statistical Analysis

Data are expressed as mean±SE. N and n correspond to the number of animals and the number of cells, respectively. Statistical significance was estimated by one-way ANOVA followed by Dunnett’s test to identify differences between groups. Differences were considered significant if $P<0.05$.

### Results

#### Ang II Is Involved in the Stenosis-Induced Increase in $I_{\text{CaT}}$ Density

We have used a model of stenosed rat treated with losartan to examine the putative effect of Ang II in the reexpression of $I_{\text{CaT}}$. After 6 weeks of stenosis, although left ventricular cells were hypertrophied by 52% compared with control cells (234±6 pF, $n=10$ versus 154±29 pF, $n=10$; $P<0.01$), no $I_{\text{CaT}}$ was detected at this time. In long-term stenosis AS group (12 weeks), the cell membrane capacitance increased by 1.5-fold as in 6-week-stenosed group (Table 1). Two weeks of losartan treatment decreased heart weight/body weight ratio without affecting cell size (AS/L versus AS). As it has

### Table 1. Characteristics of Experimental Animals

<table>
<thead>
<tr>
<th></th>
<th>BW, g</th>
<th>HW, mg</th>
<th>HW/BW, mg/g</th>
<th>Cell Capacitance, pF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTL (N=9)</td>
<td>458±6</td>
<td>1530±40</td>
<td>3.35±0.09</td>
<td>199±9 (N=32)</td>
</tr>
<tr>
<td>AS (N=12)</td>
<td>455±13</td>
<td>2340±70*</td>
<td>5.18±0.23*</td>
<td>314±12* (n=48)</td>
</tr>
<tr>
<td>AS/L (n=10)</td>
<td>467±15</td>
<td>1990±100†</td>
<td>4.23±0.10†</td>
<td>303±10* (n=39)</td>
</tr>
</tbody>
</table>

BW indicates body weight; HW, heart weight; HW/BW, heart weight/body weight ratio; CTL, sham-operated; AS, abdominal aortic stenosis; AS/L, abdominal aortic stenosis plus losartan treatment; N, number of animals; and n, number of cells.

*P<0.001 vs CTL; †P<0.01 vs AS.
been shown that losartan treatment prevents increase in the interstitial collagen fraction in rat heart after aortic stenosis, the decrease in hypertrophy in AS/L rats was probably due to a decrease in interstitial fibrosis.

After 12 weeks of stenosis, \( I_{CaT} \) was recorded in AS cells (Figures 1A and 1B). After losartan treatment, \( I_{CaT} \) is still expressed (Figure 1C) but with a significantly reduced density (0.20 ± 0.03 pA/pF, \( n=27 \) in AS/L cells and 0.40 ± 0.05 pA/pF, \( n=26 \) in AS cells; \( P<0.01 \)) (Figure 2A). We have checked that in sham-operated losartan group, neither the procedure nor the 2 additional weeks have any effect on \( I_{CaT} \) (0.41 ± 0.04 pA/pF, \( n=9 \)). We have also tested that \( I_{CaT} \) density is insensitive to acute application of losartan (2 μmol/L) in freshly isolated hypertrophied myocytes (0.44 ± 0.06 pA/pF, \( n=6 \)). Then the reduction of \( I_{CaT} \) density in AS/L results from AT1 blockade by losartan. As neither activation nor steady-state inactivation parameters of \( I_{CaT} \) were modified after losartan treatment (Figure 2B and Table 2), it can be postulated that the blockade of the AT1 pathway induced the decrease in functional channel density, leading the decrease in \( I_{CaT} \) density.

The impact of cardiac hypertrophy on L-type Ca\(^{2+}\) current (\( I_{CaL} \)) density remains unclear as previous studies have reported either a decrease or an increase of \( I_{CaL} \) density during pathology (see review\(^{27} \)). We found that neither stenosis-induced pressure overload nor losartan treatment altered \( I_{CaL} \) density in hypertrophied myocytes (9.9 ± 0.5 pA/pF, \( n=22 \), 10.9 ± 1.0 pA/pF, \( n=26 \) and 10.0 ± 0.7 pA/pF, \( n=27 \) for CTL, AS, and AS/L cells, respectively). The activation and steady-state inactivation parameters of \( I_{CaT} \) were modified after losartan treatment (Figure 2B and Table 2), it can be postulated that the blockade of the AT1 pathway induced the decrease in functional channel density, leading the decrease in \( I_{CaT} \) density.

### Table 2. Activation and Inactivation Parameters of Cardiac Calcium Currents

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>AS</th>
<th>AS/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_{50(T)} )</td>
<td>( 6.4±1.8 ) (( n=6 ))</td>
<td>( 7.4±1.6 ) (( n=8 ))</td>
<td>( 9.6±1.2 ) (( n=8 ))</td>
</tr>
<tr>
<td>( V_{50(L)} )</td>
<td>( 6.8±0.5 )</td>
<td>( 6.5±0.3 )</td>
<td>( 6.1±0.2 )</td>
</tr>
<tr>
<td>( k_t )</td>
<td>( ... )</td>
<td>( 4.1±0.4 )</td>
<td>( 5.4±0.8 )</td>
</tr>
<tr>
<td>( k_i )</td>
<td>( ... )</td>
<td>( 6.5±0.3 )</td>
<td>( 6.1±0.2 )</td>
</tr>
<tr>
<td>( V_{50(T)} )</td>
<td>( 60.5±1.1 ) (( n=19 ))</td>
<td>( 60.0±1.1 ) (( n=10 ))</td>
<td>( 60.0±1.1 ) (( n=10 ))</td>
</tr>
<tr>
<td>( V_{50(L)} )</td>
<td>( 17.2±0.6 ) (( n=19 ))</td>
<td>( 17.7±1.3 ) (( n=10 ))</td>
<td>( 17.7±1.3 ) (( n=10 ))</td>
</tr>
<tr>
<td>( k_t )</td>
<td>( ... )</td>
<td>( -6.5±0.4 )</td>
<td>( -6.3±0.3 )</td>
</tr>
<tr>
<td>( k_i )</td>
<td>( ... )</td>
<td>( -5.5±0.2 )</td>
<td>( -5.4±0.2 )</td>
</tr>
</tbody>
</table>

Values are mean ± SE; \( n \) indicates number of cells; T, T-type Ca\(^{2+}\) current; L, L-type Ca\(^{2+}\) current; \( V_{50} \), potential of half-activation or half-inactivation; \( k \), slope factor; CTL, sham-operated; AS, abdominal aortic stenosis; and AS/L, abdominal aortic stenosis plus losartan treatment.
Characteristics and Regulation of the Functional \( I_{\text{CaT}} \) Pore-Forming \( \alpha 1 \) Subunits

We tested the \( \text{Ni}^{2+} \) sensitivity of \( I_{\text{CaT}} \) in order to determine the \( \alpha 1 \) subunit–related currents induced during cardiac hypertrophy (Figure 3A). The \( \text{Ni}^{2+} \) dose-response relation indicated that \( I_{\text{CaT}} \) was blocked in a biphase manner with \( I_{50} \) of 1.6 and 240 \( \mu \text{mol/L} \). According to previous reports, 1.6 and 240 \( \mu \text{mol/L} \) \( \text{Ni}^{2+} \) sensitivity corresponds to sensitivity described for \( \text{Ca}_{\text{a},3.2} \)- and \( \text{Ca}_{\text{a},3.1} \)-related current, respectively.\(^{26,29}\) Also, the relative contribution of \( \text{Ca}_{\text{a},1} \)-related current in \( I_{\text{CaT}} \) was assessed from \( \text{Ni}^{2+} \) dose-response relation. We found that \( \text{Ca}_{\text{a},3.1} \) and \( \text{Ca}_{\text{a},3.2} \) contribute to 80% and 20%, respectively.

Interestingly, because there was no difference between \( \text{Ni}^{2+} \) dose-response curves in AS and AS/L, it appeared that the relative contributions of \( \text{Ca}_{\text{a},3.1} \) and \( \text{Ca}_{\text{a},3.2} \) subunits to \( I_{\text{CaT}} \) were not modified by losartan treatment (Figure 3A). These data indicated that the decrease in \( I_{\text{CaT}} \) density after AT\(_1\) blockade was due to a proportional decrease in the density of functional \( \text{Ca}_{\text{a},3.1} \) and \( \text{Ca}_{\text{a},3.2} \) subunits. The Ang II signaling pathway then mediates upregulation of the expression of \( \text{Ca}_{\text{a},3.1} \) and \( \text{Ca}_{\text{a},3.2} \) subunits during stenosis-induced cardiac hypertrophy.

We investigated the regulation of \( \text{Ca}_{\text{a},3.1} \) and \( \text{Ca}_{\text{a},3.2} \) expression by quantifying transcript amount (Figure 3B). Neither aortic stenosis nor losartan treatment affected the amount of \( \text{Ca}_{\text{a},3.2} \) mRNA (0.53 ± 0.07, 0.52 ± 0.06, and 0.51 ± 0.10 \( \times 10^6 \) molecules per \( \mu \text{g} \) total RNA in CTL, AS, and AS/L, respectively). As losartan treatment decreased the density of functional \( \text{Ca}_{\text{a},3.2} \) subunits, Ang II probably regulated \( \text{Ca}_{\text{a},3.2} \) channel expression at the posttranscriptional level. Conversely, the amount of \( \text{Ca}_{\text{a},3.1} \) transcripts increased by almost 3-fold in hypertrophied group (0.70 ± 0.04 versus 0.25 ± 0.09 \( \times 10^6 \) molecules in AS versus CTL cells; \( P < 0.001 \)) and was abolished by losartan treatment (0.29 ± 0.03 \( \times 10^6 \) molecules). Therefore, during stenosis-induced hypertrophy, regulation of the amount of \( \text{Ca}_{\text{a},3.1} \) mRNA is entirely dependent on Ang II signaling pathway.

Ang II Stimulates \( I_{\text{CaT}} \) Channel Expression in Cultured Newborn Cells via the MEK1/2 Pathway

We investigated the Ang II intracellular signaling events involved in the expression of the T-type channels in ex vivo model. Previous experiments have indicated that chronic Ang II stimulation failed to reexpress \( I_{\text{CaT}} \) in cultured adult ventricular cells (data not shown). Therefore, we have investigated the effect of Ang II in newborn ventricular cells that are known to exhibit basal \( I_{\text{CaT}} \).

Cultured newborn cells exhibited a typical \( I_{\text{CaT}} \) density of which was increased under Ang II stimulation (8.1 ± 0.8 pA/pF, \( n = 10 \), for Ang II and 3.6 ± 0.3 pA/pF, \( n = 8 \), for CTL; \( P < 0.001 \)) (Figures 4A and 4B). Treatment of myocytes with losartan had no effect on basal \( I_{\text{CaT}} \) density (3.8 ± 0.8 pA/pF, \( n = 10 \)) and completely blocked the Ang II–induced increase of \( I_{\text{CaT}} \) density (3.8 ± 0.4 pA/pF, \( n = 10 \); \( P < 0.001 \)) (Figure 4B). Because an AT\(_1\) receptor antagonist, PD123319, had no effect on Ang II–induced increase of \( I_{\text{CaT}} \) density (6.8 ± 0.9 pA/pF, \( n = 5 \)), then Ang II upregulates \( I_{\text{CaT}} \) density via AT\(_1\) receptor.

The increase in \( I_{\text{CaT}} \) density was not associated with changes in the activation and steady-state inactivation parameters (Figure 4C). Thus, the Ang II–induced increase in \( I_{\text{CaT}} \) density resulted from an increase in functional channel density in vitro. Interestingly, as in untreated cells, \( \text{Ni}^{2+} \) blocked \( I_{\text{CaT}} \) in a monophasic manner with an \( I_{50} \) of 260 \( \mu \text{mol/L} \) in Ang II–treated cells (Figure 4D). Thus, Ang...
II–stimulated cells exhibited Ca$_\text{v}$3.1-related current but are not potent to reexpress functional Ca$_\text{v}$3.2 subunits.

We investigated whether ERK1/2 activation plays a role in the Ang II–induced increase in I$_{\text{CaT}}$ density using MEK1/2 inhibitors (UO126 and PD98059). Peak I$_{\text{CaT}}$ density with UO126 (5.0±0.2 pA/pF, n=6) did not differ significantly from that with PD98059 (5.2±0.5 pA/pF, n=6), and both reduced the Ang II–induced I$_{\text{CaT}}$ density by 65% (Figures 5A and 5B). The reduction of I$_{\text{CaT}}$ density was due to the inhibition of Ang II effect because I$_{\text{CaT}}$ was not significantly different in cultures treated with UO126 alone or untreated (3.0±0.7 pA/pF, n=6) (Figure 5B). Because no change in the activation and steady-state inactivation characteristics of I$_{\text{CaT}}$ was observed after MEK1/2 inhibition, we can propose that the regulation of functional Ca$_\text{v}$3.1 channel density is mediated by Ang II–activated MEK1/2-dependent pathway. Use of a higher concentration of MEK1/2 inhibitor (75 μmol/L) did not further inhibit the Ang II–induced increase in I$_{\text{CaT}}$ density (5.5±0.5 pA/pF, n=5). Then the remaining I$_{\text{CaT}}$ density is related to an Ang II–activated MEK1/2-dependent pathway.

We found that the Ang II–activated signaling pathway increased the number of Ca$_\text{v}$3.1 transcripts (1.93±0.02 and 3.16±0.16×10$^6$ molecules per μg of total RNA in untreated and Ang II–treated cells, respectively; *P<0.001) (Figure 5C).

transcript amount, an additional Ang II–activated pathway also seems to be involved in this process.

**Autocrine Effect of ET on I$_{\text{CaT}}$ Channel Expression After Ang II Stimulation in Cultured Newborn Cells**

It has been reported that in cultured cardiomyocytes, Ang II increases the synthesis of ET-1, which may act as an autocrine/paracrine factor. We tested the putative autocrine effect of ET on the expression of T-type channels using multiple ET receptor antagonists: a dual ETA and ETB (bosentan), specific ETA (BQ123), and specific ETB (BQ788) (Figure 6). We found that the Ang II–induced I$_{\text{CaT}}$ density was significantly reduced by bosentan (5.4±0.3 pA/pF, n=11; *P<0.05) and BQ123 (4.8±0.4 pA/pF, n=6; *P<0.05), whereas BQ788 had no effect (8.2±0.8 pA/pF, n=8). To rule out the possibility that bosentan decreased basal I$_{\text{CaT}}$ density instead of the Ang II–increased I$_{\text{CaT}}$ density, we verified that I$_{\text{CaT}}$ density remained stable in cells treated by bosentan alone (3.9±0.3 pA/pF, n=5). These results showed that ETA stimulation mediated an autocrine ET response that participated to upregulation of I$_{\text{CaT}}$. In accordance to that, 48 hour application of ET (0.1 μmol/L) increased I$_{\text{CaT}}$ density (5.4±0.8 pA/pF, n=7 versus 3.6±0.3 pA/pF, n=8 for CTL; *P<0.05) and this increase was abolished by BQ123 (3.2±0.3 pA/pF, n=6).

The increase in I$_{\text{CaT}}$ density observed in cultured ventricular cells stimulated with Ang II reflects an additive effect of the Ang II– and ET-activated signaling pathways. Because cotreatment with bosentan and UO126 abolished the Ang II–induced in-
MEK1/2 independent ET-signaling pathway is involved in the regulation of CaV3.1. The quantification of the amount of CaV3.1 mRNA but not those of CaV3.2, (3) induced increase of CaV3.1 mRNA not alter the Ang II – AT1 increased CaV3.1- and CaV3.2-related current density in hypertrophied myocytes. We demonstrate that (1) Ang II via AT1 increased CaV3.1 expression. We show in this study that Ang II treatment leads to an increase in CaV3.1 mRNA amount can be reproduced by application of Ang II to newborn rat myocytes and thereafter blocked by losartan. Thus, Ang II–treated cultures of myocytes appear to be one relevant model for investigating the AT1 signaling pathways involved in the regulation of CaV3.1 expression. We show in this study that Ang II treatment leads to an increase in the amount of CaV3.1 mRNA, partly through ERK1/2-dependent units related to those described for fetal ventricular I_{CaT}. However, compared with fetal I_{CaT}, kinetic characteristics of I_{CaT} in hypertrophied myocytes revealed a hyperpolarized shift in the voltage-dependent activation (−39.5±0.8 mV, n=7, versus −22.4±0.6 mV, n=9, for AS and 18- and 18-day-old fetal ventricular cells, respectively), and this hyperpolarized shift was insensitive to Ang II. Because Ca2+ calmodulin– dependent protein kinase II (CaMKII) shifts the activation of I_{CaT} to more negative potentials in adrenal glomerulosa cells, 

Interestingly, after 6 weeks of stenosis, although left ventricular cells were hypertrophied, no I_{CaT} was yet reexpressed. We can note that between 6 and 12 weeks, cell membrane capacitance increased in the same way in AS than in control indicating that hypertrophy was compensated. Therefore, whereas a hypertrophic background is necessary to reexpress I_{CaT}, long-term compensatory phase of hypertrophy is required for efficient expression of T-type channels, which is likely due to the activation of a time-dependent regulator factor. Supporting that, we have observed that Ang II stimulation was inefficient to reexpress I_{CaT} in cultured adult ventricular myocytes. Therefore, further ex vivo investigation of the regulation of I_{CaT} had required myocytes potent to express I_{CaT} such as newborn myocytes.

Ang II increased the expression of T-type channel subunits via AT1-stimulated signaling pathway. Nิ3{	extsuperscript{+}} sensitivity revealed that functional CaV3.1 and CaV3.2 channels participate to an unidentified stenosis-induced factor is crucial to ensure hypertrophy-associated factor appears efficient to increase neither CaV3.2 promoter activity nor mRNA stability. It appears that the stenosis-induced increase in functional CaV3.2 proceeds through translational and/or posttranslational regulation. In newborn myocytes, CaV3.2 is also mainly regulated by translational and/or posttranslational mechanism because there is no more functional subunit while mRNA regulation persists. The blockade of functional CaV3.2 subunit is maintained in cultured newborn myocyte even after Ang II treatment. Therefore, we can propose that Ang II in addition to an unidentified stenosis-induced factor is crucial to ensure the increase of functional CaV3.2 subunit.

In contrast to CaV3.2 regulation, the amount of CaV3.1 mRNA increased during stenosis-induced hypertrophy and this increase entirely depends on the activation of AT1 signaling pathway. As a residual CaV3.1 related current persisted after AT1 blockade, we can assure that in addition to the transcriptional regulation, CaV3.1 expression is regulated through translational and/or posttranslational mechanisms. The stenosis-associated changes in I_{CaT} density and CaV3.1 mRNA amount can be reproduced by application of Ang II to newborn rat myocytes and thereafter blocked by losartan. Thus, Ang II–treated cultures of myocytes appear to be one relevant model for investigating the AT1 signaling pathways involved in the regulation of CaV3.1 expression. We show in this study that Ang II treatment leads to an increase in the amount of CaV3.1 mRNA, partly through ERK1/2-dependent

**Discussion**

This study provides data on the regulation of molecular and functional expression of T-type channels by Ang II in hypertrophied myocytes. We demonstrate that (1) Ang II via AT1 increased CaV3.1- and CaV3.2-related current density in stenosis hypertrophied myocytes, (2) CaV3.1 and CaV3.2 were differently regulated because Ang II via AT1 increased the amount of CaV3.1 mRNA but not those of CaV3.2, (3) in cultured newborn ventricular myocytes Ang II via AT1 increased I_{CaT} density and the amount of CaV3.1 mRNA through MEK1/2 pathway, and (4) increased I_{CaT} density also resulted of the autocrine action of ET released by Ang II.

Long-term aortic stenosis displayed expression of an I_{CaT} with kinetic properties and CaV3.1 and CaV3.2 pore-forming

![Figure 6. Autocrine effect of ET on Ang II–induced I_{CaT} in cultured newborn rat ventricular myocytes. A, Typical recordings are shown for cells treated with Ang II, Ang II+bosentan (Ang II+Bos), and Ang II+bosentan+UO126 (Ang II+Bos+UO). B, I_{CaT} density–voltage relationships in CTL (n=8), Ang II (n=10), Ang II+Bos (n=13), and Ang II+Bos+UO (n=6). C, Comparative I_{CaT} density recorded at −20 mV. Results are expressed as a percentage of the control current density. *P<0.05, **P<0.01, and ***P<0.001 vs Ang II; £P<0.05, ££P<0.01, and £££P<0.001 vs CTL.](http://circres.ahajournals.org/content/1246/12/1246)
activation. These data are consistent with a large body of study implicating the MAPK cascade in hypertrophic responses.\textsuperscript{19} It should be noted that ERK1/2 is activated by stenosis-induced cardiac pressure overload\textsuperscript{33} and that the MEK/ERK pathways have been shown to be involved in the reexpression of fetal genes in hypertrophic background.\textsuperscript{34} It seems reasonable to postulate that the stenosis-induced regulation of the amount of CaV 3.1 mRNA is mediated by Ang II activated MEK/ERK pathway. Further studies are required to evaluate the role of Ang II–activated ERK1/2 in the regulation of T-type channel expression in pathological situations.

It was previously shown that some of the cardiovascular hypertrophic effects of Ang II result from the autocrine/paracrine release of ET.\textsuperscript{30,35} Consistent with this, we demonstrated that the effect of Ang II stimulation on I\textsubscript{CaT} density is decreased by ETA receptor blockade in cultured myocytes. Both ET-1 and Ang II activate similar G-protein–coupled receptor signaling pathways, including those activating ERK1/2.\textsuperscript{36} Another important finding is that the blockade of ET signaling pathways did not affect the transcriptional regulation of CaV 3.1, neither in Ang II–induced ET autocrine effect nor in chronic application of ET. Therefore, ET signaling pathway regulates functional CaV 3.1 protein density through a translational and/or posttranslational mechanism. Finally, coblockade of ERK1/2 and ETA decrease more strongly I\textsubscript{CaT} density than ERK1/2 blockade alone suggesting that ET regulates CaV 3.1 through an independent MEK/ERK pathway. This indicates that Ang II and ET use different signaling mechanisms for I\textsubscript{CaT} expression in cultured myocytes.

It is not surprising that several hypertrophic factors regulated T-type channels because the blockade of Ang II effect did no wholly abolished I\textsubscript{CaT} in stenosed rats. As no more transcriptional upregulation of channel subunits occurs after losartan treatment, the residual I\textsubscript{CaT} likely results from translational and/or posttranslational mechanism. Cultured newborn cell data lead us to propose that this latter mechanism can be ensured by ET signaling pathways activation.

This study provides new evidence that Ang II is closely related to electrophysiological remodeling process that occurs during cardiac hypertrophy. In addition to I\textsubscript{CaT}, another inward current, I\textsubscript{f}, has been shown to be upregulated by Ang II during cardiac hypertrophy.\textsuperscript{37,38} As I\textsubscript{CaT} and I\textsubscript{f} are both considered to ensure pacemaker activity\textsuperscript{39,40} alterations in I\textsubscript{CaT} and I\textsubscript{f} density likely contribute to the increased propensity of the hypertrophied heart to develop arrhythmia.\textsuperscript{27} In addition to its involvement in abnormal electrical properties, I\textsubscript{CaT} has been shown to contribute to Ca\textsuperscript{2+}–dependent hormone secretion.\textsuperscript{41,42} It would therefore seems to be important to consider the biological role of I\textsubscript{CaT} with respect to pathogenesis of Ca\textsuperscript{2+} overload effects\textsuperscript{4} such as the activation of Ca\textsuperscript{2+}–dependent transduction pathways required after long-term compensate cardiac hypertrophy.

In conclusion, we found that Ang II through AT\textsubscript{1}-activated MEK-dependent pathway is responsible for the regulation of CaV 3.1 transcription (promoter activity and/or mRNA stability), whereas ET through ETA-activated MEK-independent pathway is implicated in CaV 3.1 posttranscriptional regulation (translational and/or posttranslational mechanism). Unfortunately, CaV 3.2 is not expressed in cultured myocytes that avoid detailed description of mechanisms involved in its regulation. However, we show that CaV 3.2 is only regulated at posttranscriptional level and this regulation is also mediated by Ang II. Altogether these regulations of the channel subunits lead to I\textsubscript{CaT} expression that contributes to cardiac electrophysiological remodeling. Future studies will be needed to precisely explain the relationship between I\textsubscript{CaT} expression and pathophysiological events.

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**References**

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