Independent Regulation of Cardiac Kv4.3 Potassium Channel Expression by Angiotensin II and Phenylephrine

Ting-Ting Zhang, Koichi Takimoto, Alexandre F.R. Stewart, Chongxue Zhu, Edwin S. Levitan

Abstract—Hypertrophied cardiac myocytes exhibit prolonged action potentials and decreased transient outward potassium current (Ito). Because Kv4.3 is a major contributor to Ito, we studied regulation of its expression in neonatal rat cardiac myocytes in response to the known stimulators of cardiac myocyte hypertrophy, angiotensin II (Ang II) and phenylephrine (PE). RNase protection assays and immunoblots revealed that Ang II and PE each downregulate Kv4.3 mRNA and protein. However, although PE induces a faster and more extensive hypertrophic response than Ang II, the PE effect on Kv4.3 mRNA develops slowly and is sustained, whereas Ang II rapidly and transiently decreases Kv4.3 mRNA expression. Turnover measurements revealed that Kv4.3 mRNA is very stable, with a half-life >20 hours. This suggests that Ang II must destabilize the channel mRNA. In contrast, PE does not affect the rate of Kv4.3 mRNA degradation. To test for transcriptional regulation, the 5′ flanking region of the rat Kv4.3 gene was cloned, and Kv4.3 promoter-reporter constructs were expressed in cardiac myocytes. Whereas Ang II was found to have no effect on transcription, PE inhibits Kv4.3 promoter activity. Pharmacological experiments also indicate that PE and Ang II act independently to downregulate Kv4.3 gene expression. Thus, regulation of Kv4.3 gene expression is not a simple secondary response to hypertrophy. Rather, Ang II and PE use different mechanisms to decrease Kv4.3 channel expression in neonatal rat cardiac myocytes. (Circ Res. 2001;88:476-482.)

Key Words: hypertrophy ■ gene regulation ■ ion channels/membrane transport ■ angiotensin-converting enzyme/angiotensin receptor ■ physiological and pathological control of gene expression

Cardiac hypertrophy is a pathological enlargement of the heart. It is initially developed as an adaptive response to heart pressure and volume overload. However, sustained hypertrophy leads to severe consequences, with increased risk of morbidity and mortality. Many studies have shown that cardiac excitation is altered in hypertrophied myocardium from experimental animals and patients with heart failure.1 The most consistent change seen in hypertrophied myocytes is a prolongation of action potential duration.2–6 Intensive studies on mechanisms underlying this abnormal action potential revealed that the voltage-dependent, Ca2+-independent transient outward potassium current (Ito) is important in the early phase of membrane repolarization, changes in its density will alter the time course of the action potential. Thus, it is important to elucidate the mechanism by which pathological conditions reduce the Ito in cardiac myocytes.

Recent studies have demonstrated that Kv4-channel family genes encode a large fraction of Ito in cardiac myocytes.10 In particular, the Kv4.3 gene is abundantly expressed in the rat, canine, and human hearts.11 The Kv4.3 gene, when expressed in Xenopus oocytes, generates a channel with properties similar to the native Ito.10,12,13 Furthermore, application of Kv4.3 antisense oligonucleotide to myocytes significantly reduces Ito.14 Likewise, expression of dominant-negative Kv4 constructs suppresses Ito and prolongs action potential duration.15–17 Finally, recent studies showed that ventricular Kv4.3 mRNA levels decrease in hypertensive rats, as well as in failing human heart.18–20 Thus, the decreased expression of Kv4.3 gene is, at least in part, responsible for the reduced Ito found in hypertrophied and diseased hearts.

Although decreases in Kv4.3 mRNA have been observed in hypertrophied myocardium, no study has examined the mechanisms underlying this gene regulation. Therefore, we studied the hypertrophic responses of cultured neonatal rat cardiac myocytes to angiotensin II (Ang II) and α1-adrenergic receptor agonist phenylephrine (PE). These agents have been shown to increase cell size, RNA and protein synthesis, and the expression of immediate early genes (eg, c-fos, c-jun, and c-myc) and fetal genes (eg, α-skeletal actin and β-myosin heavy chain [β-MHC]).21–30 In this study, we show that PE and Ang II independently downregulate Kv4.3 mRNA and protein in cardiac myocytes. Furthermore, our results indicate that PE inhibits transcription of the Kv4.3 gene, whereas the effect of Ang II likely involves destabilization of channel...
mRNA. Thus, 2 hypertrophic stimuli use distinct mechanisms to inhibit Kv4.3 expression in neonatal rat cardiac myocytes.

Materials and Methods

Culture and Treatment of Rat Neonatal Myocytes

Neonatal rat cardiac myocytes were isolated and prepared from 1-day-old Sprague-Dawley rats, as described previously.31 Cells were fed with fresh medium every other day, except for transfections (see below). Four days after plating, myocytes were subjected to the treatment with 100 μmol/L PE (Sigma, in 100 mmol/L stock solution with 100 mmol/L vitamin C), 100 nmol/L human Ang II (Calbiochem-Novabiochem, in 100 mmol/L stock solution with 50 mmol/L acetic acid), or vehicles.

RNase Protection Assays

RNase protection assays were performed as previously described.18 Kv4.3 cDNA template was made by inserting Kv4.3 Smal-KpnI fragment (58 to 777 bp from the translation start site) into pbScript KS (+) (Stratagene). DNA templates for GAPDH and β-actin were obtained from Ambion Inc. β-MHC cDNA fragment was prepared by reverse transcription–polymerase chain reaction (PCR) using primers 5'-CACCAACCTGTCACAGTCCG-3' (nucleotides 5680–5701) and 5'-GTGGTTTCTGCCTAAGGTGCTG-3' (complementary to nucleotides 5878–5899). The amplified fragment was subcloned into pbScript KS (+). α-Adrenergic receptor 1B (α) template was generated by digesting α cDNA plasmid (in pGEM 7Z, kindly provided by Drs Kunos and Chin, Virginia Commonwealth University, Richmond, Va) with BsaHI (nucleotides 1308–2080). Protection assays for ribosomal RNA were performed with an ~5-fold molar excess of 28S RNA probe. Briefly, cold and [32P]-labeled RNA probes were prepared with a rat 28S cDNA template (Ambion). Concentration of the prepared RNA probes was determined spectrophotometrically using 1 A260=33 μg. Approximately 0.5 μg total 28S RNA probe (~100 bases in length) was included in the hybridization. Under these conditions, 28S RNA signals were linear up to at least 10 μg of total RNA. Quantitation was performed with a PhosphorImager (Molecular Dynamics). Unless specified differently, each Kv4.3 result was normalized to β-actin mRNA and expressed as the percentage compared with a matched vehicle control.

Immunoblot Analysis

Cell extracts were prepared from myocytes grown on 60-mm culture dishes, as described previously.32 Immunoblot analysis was performed after SDS-PAGE with anti-Kv4.3 antibody (1:300, Alomone Labs). Bound antibody was detected with secondary goat anti-rabbit IgG-HRP conjugate (1:3000, Bio-Rad) using enhanced chemiluminescence method (NEN Life Science). The epitope (residues 451–467 of rat or human Kv4.3) used to raise the commercial anti-Kv4.3 antibody (N-terminus, available at http://www.circresaha.org) thus, the anti-Kv4.3 antibody was used for antigen retrieval by incubating with 0.1% trifluoroacetic acid (TFA) for 30 min at room temperature. Western blotting was then performed as described previously.

Luciferase Assay

Primers 5'-CTGTTTCACTACGCAATGTAC-3' (146 to 166 bp 5' to the Kv4.3 translation start site) and 5'-GGTAGAAGTTGACACCAAACGG-3' (complementary to 272 to 294 bp from the translation start site) were used to screen a P1 rat genomic library (Genome Systems). The positive Kv4.3 clones were then mapped with various restriction digestion. Approximately 3.2 kb of BamHI fragment was subcloned into pbScript KS (+) and sequenced. Various lengths of Kv4.3 DNA fragment upstream of the coding region were subcloned into the pGL3-basic vector (Promega). On the third day after neonatal myocytes were prepared, cells were transfected with 5 μg of one of these luciferase constructs and 0.5 μg control vector PRL-TK at total DNA amount of 25 μg with salmon sperm DNA by the calcium phosphate method. After transfection, myocytes were rinsed and incubated in serum-containing medium overnight and then serum-free medium. Twenty-four hours after transfection, luciferase activities were measured using dual-luciferase reporter assay system (Promega).

Statistical Analysis

Unpaired 2-tailed t test was used for statistical analysis, with P<0.05 being considered significant. Data are presented as mean±SEM (n=3, except where specifically mentioned). An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

Results

We first examined hypertrophic responses produced by Ang II and PE in neonatal rat cardiac myocytes. Treatment for 48 hours with Ang II or PE caused myocyte hypertrophy. In our cultures, PE was more effective than Ang II at increasing the size of myocytes (data not shown). Similarly, Ang II and PE increased the amount of fetal gene β-MHC mRNA (Figure 1). Again, PE produced a faster and larger increase in β-MHC mRNA than Ang II. Thus, PE and, to a lesser extent, Ang II induce typical hypertrophic responses in the neonatal cardiac myocyte cultures used for all subsequent studies.

Ang II and PE Decrease Kv4.3 mRNA With Distinct Time Courses

RNase protection assays showed that Ang II and PE decrease Kv4.3 mRNA expression (Figures 2A and 2B). The maximum reductions produced by Ang II and PE were 48.7±2.1%
and 51.4±3.3%, respectively, when normalized to β-actin mRNA. These responses were not simply a result of the normalization, because significant decreases with 8-hour Ang II or PE treatments were also detected when signals were normalized to the number of cells plated or to total RNA (P<0.01). Furthermore, the bottom panel of Figure 2A shows that downregulation was evident when Kv4.3 mRNA was normalized to 28S ribosomal RNA (n=2). Although the 2 agonists produced similar maximum decreases in Kv4.3 mRNA levels, the time courses for mRNA downregulation were different. Ang II caused a rapidly induced and then slowly recovering decrease in Kv4.3 mRNA. In contrast, the effect of PE developed slowly and was more sustained (Figure 2B). Thus, unlike the induction of hypertrophy, Ang II acts more quickly than PE to regulate Kv4.3 mRNA expression.

Consistent with the reductions in Kv4.3 mRNA levels, immunoblot analysis revealed that Kv4.3 channel protein was downregulated by treatment with Ang II or PE for 48 to 72 hours (n=4, Figure 2C). Because previous studies on regulation of Kv channel gene expression have found that changes in total protein are accompanied by proportional changes in cell-surface channel expression,33 the downregulation of Kv4.3 channel protein in cardiac myocytes is likely to be functionally relevant.

### Ang II and PE Independently Decrease Kv4.3 Gene Expression

Mechanical stretch induces the secretion of Ang II from cytosolic granules of neonatal cardiomyocytes.34 Therefore, PE might regulate Kv4.3 gene expression by inducing autocrine release of Ang II. To test this possibility, we used the specific AT1 receptor blocker L158,809. Treatment with this drug completely prevented the Ang II–induced decrease in Kv4.3 mRNA (Figure 3A). However, the effect of PE was not altered by cotreatment with L158,809 for 8 or 24 hours (Figure 3B). Furthermore, cotreatment with PE and Ang II induced a decrease in Kv4.3 mRNA that was larger than those obtained with either agent alone (Figure 4). Indeed, downregulation of Kv4.3 mRNA induced by one agent was not affected by the presence of the other agent for either 8 or 24 hours. These results, along with the kinetic differences shown in Figure 2, suggest that Ang II and PE independently affect Kv4.3 gene expression.

#### PE Decreases Transcription of the Kv4.3 Gene

Next we tested whether Ang II and PE affect Kv4.3 gene transcription or mRNA stability. To determine the stability of Kv4.3 mRNA, the disappearance of Kv4.3 mRNA was measured by RNase protection assay. In the upper 2 panels, β-actin mRNA was used as an internal control. Bottom panel shows results from an experiment with 8-hour treatments in which 28S ribosomal RNA was used as an internal control. P indicates probe; Y, yeast RNA; and C, control. B, Time courses of Ang II–induced and PE-induced Kv4.3 mRNA downregulation (n=3 at each time point, normalization with β-actin). C, Three days after plating, cells were treated with Ang II (100 nmol/L), PE (100 μmol/L), or vehicle for 60 hours. Medium containing the agent or vehicle was changed every 24 hours. Total protein was isolated, and Kv4.3 proteins were detected using an anti-Kv4.3 antibody.
measured after the inhibition of transcription by actinomycin D. We used a known unstable \( \alpha_{1B} \)-adrenergic receptor mRNA as a positive control.\(^{35,36} \) The disappearance of Kv4.3 and \( \alpha_{1B} \)-adrenergic receptor mRNAs at various times after the drug addition was measured (Figure 5A). Unlike the rapid decline in \( \alpha_{1B} \)-adrenergic receptor mRNA, we found that Kv4.3 mRNA is very stable (\( t_{1/2} \). 20 hours) (Figure 5B). This implies that the rapid decrease in Kv4.3 mRNA produced by Ang II (ie, 50% in 8 hours) must involve mRNA destabilization. In contrast, the delayed effect of PE is consistent with transcriptional control. To test whether PE affects Kv4.3 mRNA degradation, myocytes were pretreated with PE for 24 hours and then incubated with actinomycin D in the continued presence of the agonist. Kv4.3 mRNA levels decreased at the same rate in the presence or absence of PE (Figure 5C). Therefore, PE does not alter Kv4.3 mRNA stability.

To additionally test if PE affects Kv4.3 transcription, we examined the effect of the drug on channel promoter activity. First we obtained genomic clones for rat Kv4.3 gene and identified the transcriptional start sites using RNase protection assays and 5' RACE analysis. RNase protection assays with a 688-base RNA probe encompassing the Kv4.3 translation initiation site detected single protected fragments (≈380 bp) with adult rat brain, heart, and neonatal myocyte RNAs (Figure 6A). Moreover, RACE analysis obtained single amplified products with brain and neonatal myocyte RNAs (Figure 6B). The amplified fragments were ≈186 and ≈206 bp with brain and myocyte RNAs, respectively. The sequences of these RACE products indicate that rat Kv4.3 transcription start sites in myocytes and brain are located at 54 and 34 bp upstream of the translation initiation site, respectively (Figure 6C). Because the RNA probe of the RNase protection assay contained 326 bases of the coding region, the size of protected fragment (≈380 bp) is in agreement with the transcription start site obtained from RACE analysis. Additionally, 5' primer extension analysis with brain RNA provided a result consistent with the transcription start point obtained from 5' RACE and RNase sequences.
Ang II–treated and PE-treated cells were 96.6% and 69.8±3.8% of vehicle-treated cells, respectively. Treatment with Ang II for 3 hours or PE for 11 hours produced similar results. Thus, PE, but not Ang II, significantly decreases luciferase activity driven by the Kv4.3 promoter region (P<0.01). Taken together, it seems that PE mainly affects transcription of the Kv4.3 gene, whereas Ang II likely changes stability of channel mRNA.

**Discussion**

One of the most common findings in hypertrophied hearts is action potential prolongation attributable to reduced transient outward K⁺ current (Iₒ). This abnormal cellular electrical property is potentially arrhythmogenic and may accelerate the progression toward heart failure. Directly studying the mechanisms underlying action potential prolongation with hypertrophy in human heart is not experimentally feasible. Therefore, we took advantage of the facts that Kv4.3 is a known Iₒ subunit and that disease-associated changes in gene expression produced in human heart can also be produced by Ang II and PE in neonatal rat cardiac myocytes. We explicitly showed that PE inhibits Kv4.3 promoter activity, whereas Ang II did not. Hence, hypertrophic stimuli activate multiple independent mechanisms to decrease expression of Kv4.3 Iₒ channels in neonatal rat cardiac myocytes.

We explicitly showed that PE inhibits Kv4.3 gene transcription. Although less direct, the simplest conclusion from our data is that Ang II destabilizes Kv4.3 mRNA. No effect of Ang II on promoter activity was observed. Furthermore, it acted too quickly to be explained by transcriptional regulation. However, it should be noted that the transient kinetics of Ang II action precluded direct steady-state measurements of a change in mRNA half-life. Furthermore, the promoter activity driven by a small 5' flanking region of the Kv4.3 gene may not fully reflect its transcription in native cells. Also, directly testing for an effect of Ang II on channel gene transcription using nuclear run-on assays is extremely difficult because of the low expression of Kv4.3 gene in myocytes. Therefore, our data do not eliminate the possibility that a reduction in channel gene transcription contributes to a later phase of Ang II action. Furthermore, elucidating the mechanism of rapid Kv4.3 mRNA destabilization will require cloning of sequences required for the Ang II effect. Nevertheless, we can conclude that the action of Ang II, in contrast to PE, cannot be solely explained by a change in Kv4.3 transcription.
Although it is known that cellular excitability and ion channel expression are altered in the hypertrophied heart, the causal relationship between hypertrophy and electrical changes has been unclear. For example, it is unknown whether cardiac hypertrophy is secondary to membrane action potential prolongation. Alternatively, abnormal cellular excitability may accelerate development of hypertrophy. Previous studies showed that Kv4.2 protein level is lowered in neonatal myocytes after incubation with PE, endothelin-1, or insulin-like growth factor for 3 days. This could be interpreted to imply that the decrease in Kv4.2 protein is secondary to hypertrophy. However, abnormal action potential duration and decreased Kv4.3 mRNA can be seen with heart failure in the absence of cardiac hypertrophy. Furthermore, our results with neonatal myocytes revealed reductions in Kv4.3 mRNA within several hours, a period before the appearance of hypertrophy. In addition, we found that PE induces hypotrophic responses with a faster onset and to a larger degree than Ang II. Yet Ang II downregulated Kv4.3 mRNA much earlier than PE. Finally, the 2 hypotrophic stimuli use different mechanisms to control Kv4.3 expression. All these findings suggest that downregulation of Kv4.3 gene expression is not secondary to hypertrophy in vitro. Rather, Kv4.3 gene regulation is a part of an early response produced by conditions that can later induce neonatal rat cardiac myocyte hypertrophy.

Our studies with cultured rat neonatal myocytes pose the question of whether distinct mechanisms involved in regulation of cardiac Kv4.3 channel gene expression could differentially participate in controlling excitability under physiological and pathological conditions in adult animals and humans. On the one hand, although the baseline levels of some individual channel mRNAs change during development, mechanisms that regulate gene expression may be conserved. This seems especially likely for Kv4.3, because the mRNA level of this channel is similar in the cultures studied here and in adult rat left ventricle (K. Takimoto, unpublished data, May 2000). Furthermore, it is well established that changes in gene expression found with neonatal myocyte hypertrophy in vitro occur with cardiac hypertrophy in adult human heart. Thus, it is possible that the 2 mechanisms described here are relevant to disease states in the fully mature heart. On the other hand, it is also possible that the molecular mechanisms identified in cultured neonatal rat cardiac myocytes do not operate in nonrodent species that have a less-proximate transient outward current or at later stages of development, after channel expression and the action potential waveform have changed. At the very least, additional studies on adaptive and maladaptive changes of cell excitability with cardiac hypertrophy in humans can be guided by and compared with the hypertrophic changes in Kv4.3 gene transcription and message stability demonstrated in an experimentally accessible model system.

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Supplemental Methods

Myc-Kv4.2 expression vector was constructed by subcloning a full length rat Kv4.2 cDNA into pCS2+MT, which contains six MYC epitopes in front of the polylinker. Briefly, Kv4.2 cDNA clone (RK5, Roberds and Tamkun) was digested with BclI site in the 5' untranslated region, blunt-ended with Klenow, and cut with XhoI to liberate a fragment containing a full length Kv4.2 cDNA. The obtained fragment was then ligated with into Stul-XhoI site of pCS2+MT. The resulted construct contains the 11 amino acids RITLVVTSTSDN between six MYC tags and Kv4.2 polypeptide. The Kv4.3 expression vector has been described previously (Yang et al., JBC in press 2000, available at the JBC web site). For expression of native Kv4.2, the RK5 clone was inserted into pcDNA3 expression vector.

HEK 293 cells were grown on an 100-mm dishes and transfected with 5 μg of each K+ channel expression construct using calcium phosphate precipitation (Life Technology). Two days after transfection, Triton extract was prepared from nuclear-free membrane fraction, as described previously (Takimoto et al., Neuron 93, Circ Res 94). Immunoblot analysis was performed following SDS polyacrylamide gel electrophoresis with anti-Kv4.3 antibody (purchased from Alomone), anti-Kv4.2 antibody (purchased from Chemicon or kindly provided by Dr. J. Trimmer), or polyclonal anti-MYC antibody (MBL).

Legend for Supplemental figure

HEK293 cells were transfected with myc-tagged Kv4.2 or Kv4.3 cDNA, or without any K+ channel cDNA (Mock). Approximately 10 and 40 μg proteins were separated on a 7.5% SDS gel for blot with anti-Kv4.3 (left) and anti-MYC (right) antibodies, respectively. Note that anti-Kv4.3 antibody specifically detects two bands with ~72 KDa in addition to larger aggregates only in Kv4.3 cDNA-transfected cells. Kv4.2 is not detected by the anti-Kv4.3 antibody. Similar results were obtained with native Kv4.2. In the latter case, Kv4.2 expression was verified with two different anti-Kv4.2 antibodies. Furthermore, functional Kv4 channels were detected by patch clamping cells cotransfected with GFP. Therefore, the anti-Kv4.3 antibody is specific and does not detect Kv4.2.
Blot w/ Anti-Kv4.3

Blot w/ Anti-MYC