Circadian Variation of Cardiac K⁺ Channel Gene Expression

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Background—Many cardiac arrhythmias have their own characteristic circadian variations. Because the expression of many genes, including clock genes, is regulated variably during a day, circadian variations of ion channel gene expression, if any, could contribute to the fluctuating alterations of cardiac electrophysiological characteristics and subsequent arrhythmogenesis.

Methods and Results—To examine whether cardiac K⁺ channel gene expression shows a circadian rhythm, we analyzed the mRNA levels of 8 Kv and 6 Kir channels in rat hearts every 3 hours throughout 1 day. Among these channels, Kv1.5 and Kv4.2 genes showed significant circadian variations in their transcripts: a 2-fold increase of Kv1.5 mRNA from trough at Zeitgeber time (ZT) 6 to peak at ZT18 and a completely reverse pattern in Kv4.2 mRNA (a 2-fold increase from trough at ZT18 to peak at ZT6). Actually, along with the variations in the immunoreactive proteins, the density of the transient outward and steady-state currents in isolated myocytes and the responses of atrial and ventricular refractoriness to 4-aminopyridine in isolated-perfused hearts showed differences between ZT6 and ZT18, a circadian pattern comparable to that of Kv1.5 and Kv4.2 gene expression. Reversal of light stimulation almost inverted these circadian rhythms, although pharmacological autonomic blockade only partially attenuated the rhythm of Kv1.5 but not of Kv4.2 transcripts.

Conclusions—Among all the cardiac K⁺ channels, Kv1.5 and 4.2 channels are unique in showing characteristic circadian patterns in their gene expression, with Kv1.5 increase during the dark period partially dependent on β-adrenergic activities and Kv4.2 increase during the light period independent of the autonomic nervous function. (Circulation. 2003; 107:1917-1922.)

Key Words: circadian rhythm ■ ion channels ■ genes ■ electrophysiology

Any variables within biophysical systems show peaks and troughs in the course of a 24-hour period. Most cardiac arrhythmias also follow this rule, showing characteristic circadian variations in their occurrences.¹⁻⁵ Ventricular tachyarrhythmias are known to occur more frequently in the daytime than at night.¹⁻³ On the contrary, we have reported that paroxysmal atrial fibrillation has a nocturnal-predominant circadian pattern in its existence.⁴⁻⁵ Also, in patients with Brugada syndrome, syncope frequently occurs in the middle of the night.⁶

These circadian fluctuations in the occurrences of arrhythmias have been thought to result from the physiological responses to autonomic nervous function.⁷ Actually, the facts that β-adrenergic stimulation makes it feasible to induce ventricular tachyarrhythmias during electrophysiological study⁸ and that the circadian pattern of the ventricular arrhythmias resembles that of sympathetic activation¹⁻³ support this hypothesis. The night-predominant circadian pattern of paroxysmal atrial fibrillation and ventricular tachyarrhythmia in Brugada syndrome may be explained by the fact that parasympathetic stimulation activates the acetylcholine-sensitive K⁺ channel or the inhibitory action on the L-type Ca²⁺ channel.⁹⁻¹⁰ However, on the basis of the recently recognized fact that the expression of many genes, including the clock genes, varies over the course of a day,¹¹⁻¹³ the hearts could be fluctuating not only physiologically but also biochemically. Actually, in mouse and rat hearts, the expression of period genes is reported to show a circadian rhythm.¹⁴,¹⁵ We therefore hypothesized that the circadian variation of cardiac arrhythmias might be attributed not only to the fluctuations in the physiological responses but also to biochemically based circadian rhythms. To investigate these biochemical fluctuations, we examined whether the gene expression of cardiac K⁺ channels, which are diverse and important for repolarization, shows circadian variations.

Methods

Experimental Animals

Sprague-Dawley female rats 10 weeks old were used in the present study. The rats were kept in a room equipped with light stimulation...
Polymerase Chain Reaction Primers Used for Amplification of Inwardly Rectifying K⁺ Channel Genes

<table>
<thead>
<tr>
<th>Channel</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kir2.1</td>
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<td>5’–GATATGACCTCCGATCGTG</td>
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<tr>
<td>Antisense</td>
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<tr>
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<td>5’–ACTAAGATGTTACTGAGTCTGTGAC</td>
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<td>5’–ACTAAGATGTTACTGAGTCTGTGAC</td>
</tr>
<tr>
<td>Kir3.1</td>
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<td>5’–TGTATGGCACTCACAGG</td>
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<tr>
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<td>5’–ATTCATCTGCCACAAGG</td>
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<tr>
<td>Kir3.2</td>
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<td>5’–AGCAAGTGCGGTGACTAGG</td>
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<tr>
<td>SUR2A</td>
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<td>5’–ACTAAGATGTTACTGAGTCTGTGAC</td>
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<tr>
<td>Antisense</td>
<td>5’–TGAATGGCCACTGACGACC</td>
<td>5’–ACTAAGATGTTACTGAGTCTGTGAC</td>
</tr>
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GenBank accession numbers: Kir2.1 (AF021137), Kir2.2 (X78461), Kir3.1 (Y12259), Kir3.4 (U01707), Kir6.2 (D86309), and SUR2A (NM013040).

between 6 AM and 6 PM for 2 weeks. The animals were killed every 3 hours from 9 AM to 9 AM of the following day, and atrial appendages, right ventricular free wall, and left ventricular apex were quickly and separately removed and frozen in liquid nitrogen.

Preparation of DNA Templates
DNA templates of the 8 voltage-dependent K⁺ channels (Kv1.2, Kv1.4, Kv1.5, Kv2.1, Kv2.4, Kv4.3, erg, and KvLQT1) and 6 inwardly rectifying K⁺ channels (Kir2.1, Kir2.2, Kir3.1, Kir3.4, Kir6.2, and SUR2A) known to be expressed in rat hearts were prepared by reverse transcription–polymerase chain reaction. The primers for the voltage-dependent K⁺ channels were shown in our previous report, and those specific for the inwardly rectifying K⁺ channels are shown in the Table. The amplified cDNA fragments were subcloned into pCR II vectors (Invitrogen) and confirmed by sequencing, which was used to synthesize the antisense digoxigenin-labeled RNA probes.

RNA Preparation and RNase Protection Assay
The levels of the cardiac K⁺ channel mRNAs were assayed by use of the RNase protection assay with an RPA III kit (Ambion). Amounts of 5 to 10 μg of RNA were used for hybridization, RNase digestion, and recovery of protected RNAs and transferred to a nylon membrane after running on a denaturing gel. The membranes were incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase, and subsequently, the protected fragments were detected using the CSPD (Tropix). The chemiluminescent signals were quantified by use of the lumino-image analyzer (ATTO LightCapture AE-6960).

Western Blot Analysis
Membrane fractions of the myocardium were prepared according to the protocol by Barry et al. Membrane proteins (50 μg) were fractionated on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes, which were incubated with polyclonal primary antibody obtained from rabbits (Alamone Laboratories) and subsequently with goat anti-rabbit IgG conjugated to alkaline phosphatase.

Whole-Cell Patch-Clamp Analysis and Electrophysiological Study
To examine the roles of modified gene expression, we performed the whole-cell patch-clamp studies in isolated single myocytes and electrophysiological study in isolated-perfused whole hearts. The hearts were rapidly excised at predetermined times and retrogradely perfused with Tyrode’s solution containing (in mmol/L) NaCl 136.5, KCl 5.4, HEPES 5.5, Na₂HPO₄ 0.33, glucose 5.5, CaCl₂ 1.8, and MgCl₂ 0.53 at a pH of 7.4. After single myocytes had been isolated enzymatically from the right atrium and ventricle, whole-cell currents were recorded by the patch-clamp method with an EPC-7 patch-clamp amplifier (HEKA Elektronik) with Pulse software (HEKA Elektronik). After L-type Ca²⁺ current and Iₖ, had been eliminated by addition of 10 μmol/L nifedipine and 5 μmol/L E-4031, respectively, the densities of the transient outward and steady-state currents were determined with pipettes filled with (in mmol/L) KCl 130, MgCl₂ 1.0, HEPES 10, EGTA 5, and K₃ATP 5 (pH adjusted to 7.2 with KOH) within 2 hours after cell isolation. Contamination by Iₚ was prevented by holding the cell at −50 mV.

Electrophysiological study in the whole hearts was also performed with a conventional electrode with Langendorff apparatus. Extrasustiuli with a pulse width of 1 ms and with an output of 10 times the diastolic threshold were introduced after every 10 basic stimuli (cycle length, 150 ms). The absolute refractory periods of the atrium and ventricle were determined as the longest coupling interval of extrastimuli that could not capture the hearts.

Statistical Analysis
To examine differences in the mRNA level of the cardiac K⁺ channel genes, the value at 0 o’clock (Zeitgeber time [ZT] 18) was arbitrarily set to 1 U for quantitative comparisons. The mean values at different times in a day were compared by ANOVA. The mean values of the protein levels, the current densities, and the refractory periods were compared by unpaired t test. Statistical significance was set at a probability value of P<0.05.

Results
Circadian Variation of Cardiac K⁺ Channel mRNA Level
Among the 8 voltage-dependent K⁺ (Kv) channels and 6 inwardly rectifying K⁺ (Kir) channels, only the mRNA levels of Kv1.5 and Kv4.2 channels exhibited significant circadian variations during the course of a day. As shown in Figure 1A, the steady-state mRNA level of Kv1.5 was significantly increased during the dark period (peak at ZT18) compared with that during the light period (trough at ZT6). In contrast, the Kv4.2 mRNA level showed a complete reverse pattern, with a significant increase during the light period. These circadian variations were observed consistently in each region investigated, and the degree of the variation did not differ significantly among the atrium, right ventricular free wall, and left ventricular apex (Figure 1B). The peak values were approximately double the trough values in both of the channels. In contrast, the mRNA levels of all the other K⁺ channels (Kv1.2, 1.4, 2.1, 4.3, erg, KvLQT1, Kir2.1, 2.2, 6.2, and SUR2A/B in the atrium and the ventricle and Kir3.1/3.4 in the atrium) did not fluctuate significantly during a day (data not shown).

Circadian Variation of Kv1.5 and Kv4.2 Protein Levels
To examine the correlation between the mRNA and protein levels, Western blot analysis was performed with respect to Kv1.5 and Kv4.2 channel proteins (Figure 2). To learn whether the protein levels are maintained at stable levels...
during the day, we compared those between the midpoints during the light and dark periods (ZT6 and ZT18). Like the mRNA levels, Kv1.5 channel protein increased significantly during the dark period compared with that during the light period. The protein level of the Kv4.2 channel showed a completely reverse pattern, an increase during the light period. These results indicated that the gene expression of Kv1.5 and Kv4.2 channels did fluctuate in a day with a pattern reverse to each other, not only at their mRNA levels but also at the subsequent protein levels.

**Functional Aspects of the Circadian Variation of Kv1.5 and Kv4.2 Gene Expression**

With the assumption that the fluctuating Kv4.2 and Kv1.5 protein encodes the transient outward ($I_{\text{to}}$) and steady-state ($I_{\text{Kur}}$) currents, 19 respectively, we compared the densities of the 2 currents between ZT6 and ZT18 (Figure 3). $I_{\text{Kur}}$ density, presumably encoded by Kv1.5, was significantly larger at ZT18 than at ZT6. In contrast, $I_{\text{to}}$ density that is encoded by Kv4.2 and/or Kv4.3 tended to be larger at ZT6 than at ZT18, although the difference did not reach statistical significance because of the large cell-to-cell variations.

To examine further whether the circadian variations were functioning, we determined the absolute refractory periods of the atrium and the ventricle and compared those at ZT6 with those ZT18. In the control, the absolute refractory period did not differ significantly between ZT6 and ZT18 (atrium, 33±10 ms; ventricle, 75±5 ms). However, application of 500 μmol/L 4-aminopyridine (4-AP) to block the Kv1.5 channel 20 induced significantly different

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**Figure 1.** A, Circadian variation of Kv1.5 and Kv4.2 mRNA levels. Top to bottom, ribonuclease protection assays of atrium, left ventricle, and right ventricle. Protected fragments of Kv4.2 and Kv1.5 mRNA are shown with internal control of cyclophilin mRNA. Steady-state Kv1.5 mRNA was increased during dark period in each region compared with that during light period. In contrast, Kv4.2 mRNA level was elevated during light period. Data from 7 animals are shown in B. Kv1.5 mRNA level had a peak during dark period (at ZT15 and ZT18) and a trough during light period (at ZT3 and ZT6). Kv4.2 mRNA revealed a reverse pattern with a peak at ZT6. These differences were observed irrespective of regions.

**Figure 2.** Western blot analysis of Kv1.5 and Kv4.2 proteins of hearts taken at ZT6 and ZT18. Representative examples of left ventricular apex are shown (A). Anti-Kv1.5 antibody recognized 2 protein bands. Two bands increased similarly at ZT18 compared with those at ZT6. In contrast, Kv4.2 protein level was greater at ZT6. Data from 5 animals are shown (B). Both in atrium and ventricle, protein levels of Kv1.5 and Kv4.2 increased significantly at ZT18 and ZT6, respectively ($P<0.05$), which suggested that these protein levels were not maintained stable during the day.

**Figure 3.** A, Typical current traces from ventricular cells obtained at ZT6 (A) and at ZT18 (B). $I_{\text{to}}$ was estimated as difference between peak and end-pulse currents ($I_{\text{p}}$). $I_{\text{Kur}}$ density-voltage relationships of $I_{\text{Kur}}$ and $I_{\text{to}}$ (left, atrium; right, ventricle; n=10 each). Inset, Voltage protocol with 200-ms pulses applied at 0.1 Hz. $I_{\text{Kur}}$ density was significantly larger at ZT18 than at ZT6 in both atrium and ventricle ($P<0.05$ at voltages >0 mV). Conversely, $I_{\text{to}}$ density tended to be larger at ZT6.
circadian rhythm in the Kv1.5 gene expression. Circadian rhythms played a partial role in generating the circadian rhythm of Kv1.5 and Kv4.2 gene expression, and we found that the circadian rhythm of Kv1.5 mRNA level but not that of Kv4.2 (Figure 5). These results indicate that the circadian rhythm of the Kv1.5 channels is unique in that their gene expression exhibits significant circadian variation. (2) The circadian patterns of Kv1.5 and Kv4.2 channel gene expression were reversed to each other, with Kv1.5 predominant during the dark period and Kv4.2 during the light period. (3) These variations were reflected in the circadian variation of electrophysiological characteristics in isolated single myocytes and in whole hearts. Lastly, (4) Light stimulation played a significant role in generating these circadian rhythms, whereas Kv1.5 rhythm might be partly through β-adrenergic nervous function.

Various types of circadian variation exist in the occurrence of many arrhythmias.1–5 The distribution of ventricular tachyarrhythmias is known to have the first peak between morning and noon and the second peak in the evening.1–3 Asystole is more evenly distributed but with the primary trough at night.24 In contrast, paroxysmal atrial fibrillation exists more frequently at night.4–5 Moreover, idiopathic ventricular fibrillation with Brugada syndrome occurs more frequently at night.6 Actually, the electrophysiological characteristics of the myocardium are known to fluctuate during a day.25–28

These circadian variations of cardiac electrophysiological events and characteristics have been attributed to the physiological responses to the autonomic nervous system.7,29 Adrenergic stimulation acutely augments the L-type Ca2+ current and delayed rectifier K+ current, modifying the action potential duration.30 Cholinergic stimulation antagonizes this adrenergic stimulus and also, in the atrium, induces acetylcholine-sensitive K+ current, thereby shortening the atrial action potential.9,10 In addition, this autonomic control

**Discussion**

The major findings of the present study are as follows. (1) Among all the cardiac K+ channels, Kv1.5 and Kv4.2 channels were unique in that their gene expression exhibited significant circadian variations. (2) The circadian patterns of Kv1.5 and Kv4.2 channel gene expression were reversed to each other, with Kv1.5 predominant during the dark period and Kv4.2 during the light period. (3) These variations were reflected in the circadian variation of electrophysiological characteristics both in isolated single myocytes and in whole hearts. Lastly, (4) Light stimulation played a significant role in generating these circadian rhythms, whereas Kv1.5 rhythm might be partly through β-adrenergic nervous function.

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should alter the heart rate to result in the modification of many rate-dependent currents.

However, recent studies about the clock genes have provided a new insight into the circadian rhythm. Expression of many genes fluctuates during a day. Although most evidence has been accumulated in the gene expression of the brain, a few recent data are available from that in the heart. By investigating cardiac K⁺ channel gene expression, the present study is the first to demonstrate that the expression of cardiac ion channels indeed fluctuated during the course of a day. Among 14 cardiac K⁺ channel genes, the expression of Kv1.5 and Kv4.2 exhibited significant circadian variations that were the reverse of each other, whereas that of the other 12 K⁺ channels was maintained stable. These facts added to the past circadian rhythm of cardiac electrophysiology a new concept, that the electrophysiological variables related to Kv1.5 and Kv4.2 channels could be modulated with a daily cycle on a biochemical basis as well. Actually, the density of the steady-state current presumably encoded by Kv1.5 was significantly different between ZT6 and ZT18. Also, that of the transient outward current partially encoded by Kv4.2 tended to be larger at ZT18 than at ZT6. Moreover, the electrophysiological studies in the whole hearts supported these results. The atrial and ventricular refractory periods did not respond uniformly to low and high concentrations of 4-AP between light and dark periods. All of these results indicated that the biochemical substrates for the electrophysiological properties were not maintained stable during a day and thus were well explained by the circadian rhythm of Kv1.5 and Kv4.2 gene expression.

The mechanisms underlying the circadian variation of Kv1.5 and Kv4.2 gene expression also deserve discussion. Just as light is known to play an important key role in generating the biochemical clock in the central nervous system or eye, this was also the case in the Kv1.5 and Kv4.2 circadian rhythm in the heart, although it remains unknown whether the effects were direct. Possibly, the results with pharmacological blockade would suggest an indirect effect via β-adrenergic nervous function in Kv1.5 circadian rhythm. However, the rhythms of both genes could be a result of multiple factors, including the circadian clock in the heart, nutritional status during the day, diurnal variations in circulating hormones, and changes in autonomic nervous function. The present study could not differentiate between these influences. In fact, the effects of propranolol on Kv1.5 mRNA circadian rhythms could also be indirect, because the autonomic nervous system can affect various other humoral factors, which in turn affect heart gene expression.

Although the electrophysiological variables related to Kv1.5 and Kv4.2 channels could fluctuate during a day, it remains unknown whether these circadian variations could be related to those of cardiac arrhythmias. The present results, however, would promote the premise that the circadian variation of cardiac arrhythmias could result from the net effects of physiological responses and also biochemical variations. In fact, it is fascinating that the cardiac arrhythmias originating from tissues with prominent Iₒ encoded by Kv4.2/4.3 channels, ie, paroxysmal atrial fibrillation from the atrium and Brugada syndrome from the right ventricular outflow tract, have a nocturnal-dominant circadian variation. At night, the increased gene expression of the Kv4.2 channel would shorten the action potential on a biochemical basis, and moreover, elevated muscarinic stimulation physiologically would also enhance this action potential shortening by depressing L-type Ca²⁺ current and/or inducing acetylcholine-sensitive K⁺ current. The sum of these physiological and biochemical effects of action potential shortening should serve as a factor for facilitating reentry. At present, however, it is difficult to discriminate between the physiological and biochemical backgrounds.

There are several limitations in the present study. First is the species difference. Rats are nocturnal animals, unlike human beings, and might show a totally different circadian rhythm. Second, although 4-AP inhibits Kv1.5 at a low concentration and Iₒ at a high concentration, the pharmacological discrimination between these channels would make it inappropriate to be definitely determined. Last, Western blotting and electrophysiological studies were compared between the midpoints during the light and dark periods because their small fluctuating ranges made them difficult to evaluate precisely with frequent samplings. Therefore, it is unknown whether the protein and electrophysiological properties also have circadian rhythms similar to the transcript levels. With these limitations, the present study would provide an aid for reconstructing the concept of circadian rhythm of cardiac electrophysiology. Kv1.5/4.2 channels have the circadian rhythm of their gene expression and thus could be involved in the mechanisms underlying the circadian rhythm of cardiac electrophysiology.

References


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