Inotropic Response of Cardiac Ventricular Myocytes to β-Adrenergic Stimulation With Isoproterenol Exhibits Diurnal Variation

Involvement of Nitric Oxide

Helen E. Collins, Glenn C. Rodrigo

Rationale: Although >10% of cardiac gene expression displays diurnal variations, little is known of their impact on excitation–contraction coupling.

Objective: To determine whether the time of day affects excitation–contraction coupling in rat ventricles.

Methods and Results: Left ventricular myocytes were isolated from rat hearts at 2 opposing time points, corresponding to the animals resting or active periods. Basal contraction and [Ca\(^{2+}\)]\(_i\) was significantly greater in myocytes isolated during the resting versus active periods (cell shortening 12.4±0.3 versus 11.0±0.2%; P<0.05 and systolic [Ca\(^{2+}\)]\(_i\) 422±12 versus 341±9 nmol/L; P<0.01. This corresponded to a greater sarcoplasmic reticulum (SR) Ca\(^{2+}\) load (672±20 versus 551±13 nmol/L P<0.001). The increase in systolic [Ca\(^{2+}\)]\(_i\) in response to isoproterenol (>3 nmol/L) was also significantly greater in resting versus active period myocytes, reflecting a greater SR Ca\(^{2+}\) load at this time. This diurnal variation in response of Ca\(^{2+}\)-homeostasis to isoproterenol translated to a greater incidence of arrhythmic activity in resting period myocytes. Inhibition of neuronal NO synthase during stimulation with isoproterenol, further increased systolic [Ca\(^{2+}\)]\(_i\) and the percentage of arrhythmic myocytes, but this effect was significantly greater in active period versus resting period myocytes. Quantitative RT-PCR analysis revealed a 2.65-fold increase in neuronal NO synthase mRNA levels in active over resting period myocytes (P<0.05).

Conclusions: The threshold for the development of arrhythmic activity in response to isoproterenol is higher during the active period of the rat. We suggest this reflects a reduction in SR Ca\(^{2+}\) loading and a diurnal variation in neuronal NO synthase signaling. (Circ Res. 2010;106:1244-1252.)

Key Words: E-C coupling ■ β-adrenergic ■ diurnal ■ nitric oxide synthase ■ cardiomyocyte

The existence of a biological circadian rhythm is thought to help prepare animals for the switch from a foraging “active period” to a sleeping “rest period.” The cardiovascular system is central to normal functioning of the individual and it is not surprising that strong diurnal variations are found in many hemodynamic parameters, with a morning peak in stroke volume, heart rate and blood pressure in man\(^{1,2}\) corresponding to a night peak in nocturnal animals.\(^3\) In the cardiovascular system, these cycles were originally thought to be driven by external influences, such as an increase in circulating epinephrine and norepinephrine in the morning, reflecting diurnal patterns of sympathetic activity that also peak early in the morning.\(^4,5\) More recently, the myocardial cells have been shown to possess the key molecular elements that constitute the circadian clock,\(^6,7\) and it has been proposed that much of the diurnal variation in ventricular function reflects variation in gene transcription regulated by a complex interaction of extracellular neurohumoral influences and the central and peripheral circadian clock.\(^8,9\) Indeed, >10% of rat cardiac genes are now known to show diurnal variation.\(^10\)

The morning prevalence of many pathological cardiovascular events is well documented, and it is possible that the diurnal variations in gene transcription and the resulting impact on cardiac function may contribute to the morning peak in these events.\(^8,9\) For example, the risk to development of congestive heart failure increases in patients who have their myocardial infarction early in the morning,\(^11,12\) possibly reflecting an increased sensitivity to oxidative stress and Ca\(^{2+}\) regulation, and the incidence of sudden cardiac death resulting from ventricular arrhythmias, exhibits a similar circadian variability as sympathetic activity.\(^13,14\) Young and colleagues have shown a

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strong diurnal pattern of gene expression of many key metabolic enzymes in the heart that displays a similar pattern as the intrinsic circadian clock and genes. Furthermore, they linked this variation in metabolic enzymes with a noticeable peak in power, carbohydrate oxidation and oxygen consumption during the active period of rats. However, less is known about the impact of the diurnal rhythms on excitation–contraction (E-C) coupling mechanisms.

We have therefore set out to determine whether there are any diurnal variations in E-C coupling in rat ventricular myocytes with particular reference to Ca\textsuperscript{2+}-homeostasis. As there is strong diurnal variation in sympathetic activity, which may impact on arrhythmia generation in disease conditions, we also looked at the impact of \(\beta\)-adrenergic stimulation on contraction and L-type Ca\textsuperscript{2+} current density and its impact in arrhythmia generation.

**Methods**

Adult male Wistar rats (~300 g) were housed in environmentally controlled rooms (12-hour light/dark cycle) in the Division of Biomedical Services at the University of Leicester. Left ventricular myocytes were isolated either during the resting period (3 hours after lights were turned on) or the active period (3 hours after lights were turned off). The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Details of the experimental methodologies for measuring L-type Ca\textsuperscript{2+} current density, [Ca\textsuperscript{2+}]i, contraction and arrhythmia generation from single myocytes and left ventricular developed pressure (LVDP) from isolated hearts, together with quantitative RT-PCR determination of mRNA are given in the expanded Methods section, available in the Online Data Supplement at http://circres.ahajournals.org.

**Results**

**Diurnal Variation in Basal Ca\textsuperscript{2+} Transients and L-Type Ca\textsuperscript{2+} Current in Rat Ventricular Myocytes**

Many hemodynamic parameters exhibit diurnal cycling, including stroke volume, which dips during the animals rest period. To determine whether this reflects changes to E-C coupling of the ventricular muscle, we measured [Ca\textsuperscript{2+}]i in myocytes isolated during the resting and active periods of the rat, superfused with normal Tyrode and electrically stimulated at 1Hz. Paradoxically, the basal diastolic and systolic [Ca\textsuperscript{2+}], was significantly higher in the resting period than the active period myocytes, and this correlated with a greater percentage of cell shortening in resting period myocytes (Table 1). We also found that the rate of relaxation of the electrically evoked Ca\textsuperscript{2+} transient was significantly faster in the resting period myocytes. Consistent with the higher systolic [Ca\textsuperscript{2+}], and enhanced rate of relaxation of the Ca\textsuperscript{2+} transient, the resting period myocytes had a higher SR Ca\textsuperscript{2+} content, indicated by the peak Ca\textsuperscript{2+}-release in response to caffeine (Table 1). There was no difference in the rate of relaxation of the caffeine-induced Ca\textsuperscript{2+} transient, suggesting no diurnal cycling of sodium/calcium exchange (NCX) activity. However, the peak L-type Ca\textsuperscript{2+} current showed a reverse dependency on time of day, with a higher current density in active period myocytes (P<0.01).

**The Increase in the Ca\textsuperscript{2+} Transient and L-Type Ca\textsuperscript{2+} Current During Stimulation by Isoproterenol Is Greater in Myocytes Isolated during the Resting Period**

Mouse hearts exhibit a greater increase in cardiac power and efficiency to epinephrine during the active

<table>
<thead>
<tr>
<th>Table 1. Diurnal Variation in E-C Coupling Parameters in Rat Ventricular Myocytes</th>
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<tr>
<td><strong>Resting Period</strong></td>
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<tr>
<td>Diastolic calcium, nmol/L</td>
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<tr>
<td>Systolic calcium, nmol/L</td>
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<tr>
<td>Exponential time constant of relaxation of Ca\textsuperscript{2+} transient, ms</td>
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<td>Cell shortening, % cell length</td>
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<td>L-type Ca\textsuperscript{2+} current density</td>
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<tr>
<td>Peak Ca\textsuperscript{2+} release in normal Tyrode (20 mmol/L caffeine)</td>
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Diastolic and systolic calcium, exponential time constant of relaxation of Ca\textsuperscript{2+} transient and cell contraction recorded from field-stimulated ventricular myocytes and L-type Ca\textsuperscript{2+} current density recorded in voltage-clamped myocytes, isolated from rats during the resting and active period and superfused with normal Tyrode. Peak Ca\textsuperscript{2+} release was determined by the rapid application of 20 mmol/L caffeine. Means±SEM; **P<0.01, ***P<0.001, Student’s t test.
period. To determine the impact of sympathetic activity on E-C coupling, [Ca$^{2+}$]$_i$, recorded from a single ventricular myocyte isolated during the resting (top) and active period (bottom) stimulated at 1 Hz in normal Tyrode and in response to Tyrode containing ISO 10 nmol/L. B, Dose-response curves of diastolic (triangles) and systolic (circles) calcium to ISO recorded from resting (white symbols) and active period (gray symbols) myocytes. Means±SEM, 25 to 52 cells; >8 hearts. Diastolic [Ca$^{2+}$]$_i$ for resting period myocytes is obscured by active period data.

Because an increase in the L-type Ca$^{2+}$ current is in part responsible for the positive inotropic effect of ISO, albeit limited in the rat heart, we also determined the effect of ISO on basal L-type Ca$^{2+}$ current in resting period compared to 1384 ± 2330 nmol/L in active period myocytes ($P<0.01$) (Figure 1B), and this was mirrored by the peak Ca$^{2+}$-release in response to caffeine of 1177.4 ± 90.5 nmol/L in resting period compared to 837.7 ± 59.0 nmol/L in active period myocytes (19 to 24 cells; 4 hearts, $P<0.01$).

Ventricular Myocytes Isolated From Rat Hearts During the Active Period Are Less Sensitive to ISO-Induced Arrhythmias

Maximal stimulation of β-adrenergic receptors with high concentrations of ISO can result in the development of arrhythmic activity, because of afterdepolarizations. As we found a diurnal variation in the response of myocytes to ISO, we looked at the induction of arrhythmic activity in field-stimulated ventricular myocytes during exposure to increasing concentrations of ISO. Significantly more resting period than active period myocytes developed arrhythmic activity following simulation with 3, 10, 50 nmol/L ISO for 5 minutes ($P<0.05$, Figure 3). The arrhythmic activity was seen as extrasystoles occurring after relaxation had occurred, suggestive of delayed afterdepolarization-initiated extrasystoles. Indeed, in a separate set of experiments we measured electric activity from single resting period myocytes, and only detected the presence of...
delayed afterdepolarization–induced extrasystoles (Online Figure I).

NO Synthase Is Involved in the Diurnal Variation in the Response of Ventricular Myocytes to ISO

ISO is a nonselective β-adrenergic agonist that stimulates β1, -2, and -3 receptors. Whereas activation of both β1 and β2 receptors results in a positive inotropy, in part because of an increase in the L-type Ca2+ current and SR Ca2+ loading, activation of β3 receptors is known to have a negative inotropic effect through the action of NO.22,23 We therefore investigated the effects of NO synthase (NOS) inhibition with a nonselective NOS inhibitor, N([^4]S)-nitro-L-arginine (L-NNA), on the ISO-induced increase in systolic [Ca2+]i and the L-type Ca2+ current density in resting period and active period myocytes.

The increase in systolic [Ca2+]i, in response to a sub-maximal concentration of ISO (5 nmol/L) is greater in the resting period than the active period myocytes (Figures 1 and 4A). However, L-NNA (500 μmol/L) induce a further increase in systolic [Ca2+]i, which was significantly greater in active period myocytes (P<0.001, Figure 4A). In a similar manner, the L-type Ca2+ current recorded following ISO stimulation was only significantly increased by NOS inhibition in active period myocytes (Figure 4B). The data suggest an involvement of NOS signaling in the depressed response of myocytes, isolated during the active period, to ISO stimulation.

Neuronal NOS Is Responsible for the Reduced Response of Active Period Myocytes to ISO Stimulation

We performed quantitative RT-PCR analysis of the key E-C coupling genes, to determine whether levels of gene expression of key elements involved in E-C coupling might contribute to our observed differences between resting and active period myocytes. There was no significant difference in L-type Ca2+ channel, SERCA-2A, phospholamban, or ryanodine receptor 2 gene expression; whereas NCX1 and β1-adrenoreceptor were both significantly higher in active period myocytes, although in the case of NCX1 this was a modest difference (Table 2).

Because activity of both endothelial (e)NOS and neuronal (n)NOS has been linked to sympathetic stimulation through an increase in [Ca2+]i and β3-adrenoreceptor activation, we performed quantitative RT-PCR analysis of nNOS and eNOS on snap-frozen tissue from the left ventricular free-wall of hearts taken during the resting and active period of the animal. We found a significant and large difference in nNOS expression with 5.04-fold increase in expression in ventricular muscle isolated from active period compared to resting period hearts, a profile that also existed in isolated ventricular myocytes, albeit less pronounced at 2.65-fold (P<0.05, Table 2). We also found that inhibition of nNOS with the specific inhibitor N^-[4S]-4-amino-5-[(2-aminoethyl)amino]pentyl]-N'-nitroguanidine tris(trifluoroacetate) (4-AAPNT) (200 nmol/L), significantly increased the systolic [Ca2+]i, in
active period myocytes previously stimulated with ISO ($P<0.01$, Figure 5A) and increased the percentage of active period myocytes developing arrhythmic activity in the presence of 3 and 10 nmol/L ISO ($P<0.001$, Figure 5B).

### Ventricular Function of Isolated Rat Hearts Exhibits a nNOS-Dependent Diurnal Variation to ISO Stimulation

To determine whether the involvement of nNOS in the diurnal responsiveness of isolated myocytes to ISO stimulation impacts on function in the intact heart, we measured LVDP in ex vivo Langendorff perfused hearts isolated during the resting and active period of the rat, electrically paced at 4 Hz. LVDP was greater during the resting period of the animal at 79.7 ± 5.6 mm Hg compared with 69.3 ± 3.2 mm Hg in the active period, but this was not significant ($P=0.09$). However, resting period hearts were significantly more responsive to ISO (50 nmol/L; $P<0.01$). Moreover, inhibition of nNOS with 4-AAPNT (200 nmol/L) during ISO stimulation increased LVDP significantly in active period hearts ($P<0.001$; Figure 6). These data confirm that the diurnal variations we observe in isolated myocytes are reflected in the whole heart.

### Discussion

Many key variables of the cardiovascular system exhibit diurnal patterns, some of which may be attributable directly to the heart, reflecting changes in E-C coupling of the individual ventricular myocytes. Our study shows for the first time, a diurnal variation in E-C coupling of myocytes, with basal systolic [Ca$^{2+}$]$_i$ and contraction strengths significantly higher in myocytes isolated during the resting period of the nocturnal rat. However, the opposite was true for the L-type Ca$^{2+}$ current, which was significantly higher in myocytes isolated during the active period. In addition, the increase in systolic [Ca$^{2+}$]$_i$, in response ISO showed significant diurnal variation, with a greater maximal stimulation of systolic [Ca$^{2+}$]$_i$, and a greater increase in SR Ca$^{2+}$ loading, in myocytes isolated during the resting period. This was reflected by a higher percentage of resting period myocytes developing arrhythmic activity during stimulation with ISO. Furthermore, we have shown that this diurnal variation in responsiveness to β-adrenergic stimulation with ISO is dependent on nNOS activity.

### Diurnal Variation in Basal E-C Coupling

It is now believed that diurnal variations in physiological function are not simple responses of the animal reacting to

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**Table 2. Diurnal Variation in Expression of Genes Involved in β-Adrenergic Stimulation of E-C Coupling**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Tissue</th>
<th>ZT3 (Resting Period)</th>
<th>ZT15 (Active Period)</th>
<th>Fold Change (Resting vs Active Period)</th>
<th>ΔCt (vs β-Actin)</th>
<th>$P$ (From ΔCt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-type Ca$^{2+}$ channel (caca1c)</td>
<td>Left ventricle</td>
<td>5.03±0.05</td>
<td>5.17±0.12</td>
<td>NS</td>
<td>0.92</td>
<td></td>
</tr>
<tr>
<td>β1-adrenoceptor</td>
<td>Left ventricle</td>
<td>6.36±0.15</td>
<td>5.20±0.29</td>
<td>&lt;0.05</td>
<td>2.38</td>
<td></td>
</tr>
<tr>
<td>NCX1</td>
<td>Left ventricle</td>
<td>3.45±0.07</td>
<td>3.10±0.06</td>
<td>&lt;0.05</td>
<td>1.28</td>
<td></td>
</tr>
<tr>
<td>SERCA-2A</td>
<td>Left ventricle</td>
<td>−2.36±0.04</td>
<td>−2.53±0.07</td>
<td>NS</td>
<td>1.12†</td>
<td></td>
</tr>
<tr>
<td>PLB</td>
<td>Left ventricle</td>
<td>−2.03±0.04</td>
<td>−2.14±0.03</td>
<td>NS</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td>RyR2</td>
<td>Left ventricle</td>
<td>0.76±0.12</td>
<td>0.67±0.12</td>
<td>NS</td>
<td>1.09</td>
<td></td>
</tr>
<tr>
<td>NOS-3 (eNOS)</td>
<td>Left ventricle</td>
<td>6.21±0.06</td>
<td>5.94±0.10</td>
<td>NS</td>
<td>1.17†</td>
<td></td>
</tr>
<tr>
<td>NOS-1 (nNOS)</td>
<td>Left ventricle</td>
<td>14.14±0.77</td>
<td>11.52±0.64</td>
<td>&lt;0.05</td>
<td>5.04†</td>
<td></td>
</tr>
<tr>
<td>NOS-1 (nNOS)</td>
<td>LV myocytes</td>
<td>12.26±0.52</td>
<td>10.85±0.31</td>
<td>&lt;0.05</td>
<td>2.65†</td>
<td></td>
</tr>
</tbody>
</table>

Gene expression from the left ventricular free-wall collected during the resting and active periods. ΔCt values compared to β-actin expression, and fold changes in expression of active compared to resting period hearts. Data are shown as means ± SEM from 5 hearts at each time point, with $P$ values (Student's $t$ test). NOS-1 was also determined in left ventricular (LV) myocytes. Fold change calculated using the ΔΔCt method or Pfaff† correction methods (Online Data Supplement). PLB indicates phospholamban; RyR2, ryanodine receptor 2.
its environment, rather they result from a complex interplay between extracellular stimuli such as autonomic function and the intrinsic circadian clock. Our data show that the basal systolic [Ca\(^{2+}\)], and contraction strength is, paradoxically, lower in myocytes isolated during the active period of the rat. The reduction in Ca\(^{2+}\) transient during the active period was not attributable to a smaller L-type Ca\(^{2+}\) current, which was found to have a reverse diurnal pattern, peaking during the active period. The lower Ca\(^{2+}\) transient in active period myocytes is therefore likely to result from changes to SR function.

Consistent with this, we found a slower rate of relaxation of the Ca\(^{2+}\) transient in active period myocytes, suggestive of a reduction in SERCA-2A activity, which would account for the reduction in SR Ca\(^{2+}\) loading in active period myocytes. We did not find any diurnal variation in SERCA-2A, phospholamban, or ryanodine receptor 2 mRNA levels, suggesting a posttranslational modification of SR activity, possibly involving NOS signaling. Indeed, a role for endogenous NOS in basal contraction of the heart is apparent from genetic studies.\(^{24-27}\) However, the different NOS isoforms are thought to have localized effects, because they are restricted within cell.\(^{27,28}\)

For example, eNOS is restricted to the caveolae where it is thought to target L-type Ca\(^{2+}\) current\(^{29}\) and nNOS both to the SR\(^{26,30}\) and sarcolemmal.\(^{31}\) The SR has been identified as a target for nNOS signaling, with myocytes from nNOS\(^{-/-}\) exhibiting increased Ca\(^{2+}\) transient and SR Ca\(^{2+}\) load but paradoxically a reduction in the rate of decay of the Ca\(^{2+}\) transient, suggesting a reduction in SERCA activity.\(^{26,30}\)

However, in a nNOS overexpression model, contraction strength, Ca\(^{2+}\) transient amplitude and SR Ca\(^{2+}\) loading were decreased but this was coupled to a reduction in the rate of relaxation and decay of the Ca\(^{2+}\) transient.\(^{24}\) The latter findings are consistent with the findings of our study, where the rate of relaxation of the Ca\(^{2+}\) transient is reduced and SR Ca\(^{2+}\) load decreased in active period myocytes that have increased expression of nNOS (2.65-fold). Any additional effects on the NCX was also not suggested either from mRNA levels, which varied slightly raised in active myocytes, or the rate of relaxation of the caffeine-induced Ca\(^{2+}\) transient.

**Diurnal Variation in β-Adrenergic Stimulation Reflect Changes in nNOS Activity**

In addition to the diurnal variations in the basal inotropic state of myocytes, we also found a significant depression in response of the Ca\(^{2+}\) transient of active period myocytes to ISO, which was mirrored by the response of LVDP of isolated hearts, and which were both reversed by inhibition of nNOS. The inotropic response of cardiomyocytes to β-adrenergic stimulation has been shown to be modulated by endogenous NOS using gene deletion mice, although the reports vary in their findings for nNOS compared to eNOS. For example, Barouch et al\(^{32}\) report enhancement of the ISO response in eNOS\(^{-/-}\) mouse myocytes. Although, unlike many gene deletion studies since, these authors did not find any difference in basal Ca\(^{2+}\) transients and sarcomere shortening. Wang et al,\(^{32}\) also found an increased response to ISO in eNOS\(^{-/-}\) myocytes, although in this study a high concentration of 1 mmol/L ISO was used and the specific eNOS inhibitor 1-A^\(_5\)- (1-iminoethyl)-ornithine had little effect on the ISO response of wild-type myocytes. In contrast, Martin et al,\(^{33}\) describe enhancement of the contractile response to 100 nmol/L ISO in nNOS\(^{-/-}\) and no significant difference in eNOS\(^{-/-}\) mouse myocytes.

Both nNOS and eNOS are Ca\(^{2+}\)-dependent forms of NOS, and it is possible that the reduction in response that we observe in myocytes isolated during the active period is attributable to a Ca\(^{2+}\)-dependent stimulation of nNOS, which is more highly expressed at this time. However, our data for the Ca\(^{2+}\) transient were collected in the presence of intracellular BAPTA to buffer [Ca\(^{2+}\)], suggesting any modulating effect of the Ca\(^{2+}\) current by nNOS was not Ca\(^{2+}\)-dependent. ISO is a nonspecific β-adrenergic agonist, and β3-adrenergic stimulation is negatively inotropic,\(^{25}\) involving a complex interaction with the SR and L-type Ca\(^{2+}\) channel through NO-dependent pathways which have not been fully characterized.\(^{27,34}\) A link between β3-adrenergic receptor stimulation and NO-mediated reduction in responsiveness to β1/2-adrenergic stimulation has been established in β3\(^{-/-}\) mice, but this was linked to eNOS, although it was not clear whether...
nNOS was also looked at in these studies. There is growing evidence for the presence of nNOS at the sarcolemma as well as the SR, and its involvement in the chronotropic effects of β3-adrenoreceptor stimulation has been shown. However, it remains to be determined whether nNOS is also involved in the negative inotropism of β3 signaling in the heart.

Our data are not consistent with the reduced response of active phase myocytes to ISO resulting from residual adrenergic stimulation, as basal systolic [Ca2+]i, SR Ca2+ content and SERCA activity were all lower in these myocytes; however, receptor desensitization cannot be ruled out.

Diurnal Variation in β-Adrenergic Stimulation Influences Arrhythmic Response of Ventricular Myocytes to ISO

The circadian pattern of sudden cardiac death in patients with chronic heart failure peaks in the morning, and is suggested to reflect an increase in incidence of arrhythmias, possibly triggered by the peak in sympathetic activity. High or sustained levels of sympathetic stimulation can lead to the development of ventricular arrhythmias linked to afterdepolarizations, which are particularly evident in cardiomyopathies and appear to show diurnal variation. These take the form of either early afterdepolarization, resulting from reactivation of L-type Ca2+ channels, and/or delayed afterdepolarization, resulting from spontaneous release of Ca2+ by the SR. Not surprisingly, we found that myocytes develop arrhythmic activity when exposed to high concentrations of ISO comparable to the concentrations of epinephrine/norepinephrine achieved in rats during stress. Moreover, the propensity of myocytes to develop arrhythmic activity during ISO stimulation is significantly greater during the resting than active period, when they are more responsive to ISO. This increase in arrhythmic activity could reflect the greater SR Ca2+ loading and/or the increased L-type Ca2+ current density following ISO stimulation, resulting from a reduction in nNOS activity during the resting period.

The antiadrenergic effects of NO signaling have been suggested to prevent Ca2+-overload injury and prevent the induction of arrhythmias during maximal β-adrenergic stimulation. Therefore, the high nNOS levels during the animals active period when sympathetic activity is highest and subject to surges, could act to reduce the incidence of arrhythmias during periods of high stress. It remains to be seen whether diurnal variation in nNOS signaling are also present in other species, including man.

Significance of This Study

Abnormal function of the intrinsic circadian clock and diurnal cycling of some cardiac genes has been observed to occur in pathophysiological conditions such as pressure-induced hypertrophy, myocardial ischemia and diabetes. It is not clear whether diurnal variation in NOS and E-C coupling genes are altered in disease. However, if the diurnal variation in nNOS expression and activity that we have seen is altered in diseased myocardium, either blunted or advanced in time, this could render the heart prone to sympathetic-induced arrhythmias, because the normal surges in sympathetic activity are likely to still occur because of environmental cues such as daylight and activity. Interestingly, recent genome-wide association studies have identified the nNOS adapter protein NOS1AP (CAPON) as a regulator of cardiac repolarization and that sequence variations in CAPON are associated with baseline QT interval and subsequent risk for sudden cardiac death. It is possible that in addition to this genetic determinant of sudden cardiac death, a diurnal influence could result from nNOS signaling.

Limitation of This Study

Our findings that basal inotropy and responsiveness to ISO are greater during the resting period are at odds with the literature. For example, the diurnal variation in response to increased workload and epinephrine in mouse hearts was greatest during the active period. However, this was in response to 1 μmol/L epinephrine and HR was not controlled increasing from 300 to >500 bpm. At these very high frequencies LVDP in the working heart will be sensitive to ventricular relaxation and filling governed by the duration of diastole. Yamashita et al. have shown a trough in Kv4.2 expression in the resting period, which would increase the action potential duration and reduce diastolic interval at very high heart rates. This could mean that the hearts are less efficient at very high HR during the animal’s resting period. Alternatively, this difference may simply reflect species and/or drug variation.

It is possible that diurnal variations in cellular chemistry render the cells more sensitive to modifications during the isolation process, which could account for some of our observations, or that the isolation process alters the intrinsic circadian clock. However, quantitative RT-PCR data show that the isolated myocytes exhibit similar cycling of the circadian genes; Clock and Per2 and of nNOS (Online Figure II) and the sensitivity to ISO is also observed in Langendorff perfused rat hearts. In addition, our cells do respond in a similar fashion as isolated tissue to many physiological stimuli (Online Data Supplement).

Our study looked at only 2 time points and therefore does not represent a complete picture of diurnal cycling. However, this study was designed to identify significant differences between active and resting animals. As such, these times represent periods when the animal’s behavior has fully adapted to their active or resting state. In addition, we found no difference in the E-C coupling parameters measured in myocytes between 0 to 4 hours or 4 to 8 hours after isolation, suggesting that the intrinsic clock did not continue to influence gene transcription.

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Disclosures

None.

References


Novelty and Significance

**What Is Known?**
- Many hemodynamic parameters exhibit strong dependence on time of day, peaking in the early morning when sympathetic activity is high.
- Sympathetic activity enhances ventricular function via $\beta_1$- and $\beta_2$-adrenoreceptor activation, but excessive sustained stimulation can induce arrhythmias.
- Sympathetic stimulation of $\beta_3$-adrenoreceptors depresses ventricular function and protects against sympathetic driven arrhythmias.

**What New Information Does This Article Contribute?**
- In rat ventricular myocardium, the calcium transient and resulting contraction, as well as the response to isoproterenol, exhibit a strong dependence on the time of day.
- The increase in the calcium transient in response to isoproterenol is greatest during the resting period of the rat, which parallels diurnal variation in NO signaling.
- This variation in responsiveness of the rat ventricular myocytes to $\beta$-adrenergic stimulation increases the incidence of arrhythmic activity in response to sympathetic stimulation.

Whereas as many as 10% of rat cardiac genes have been shown to exhibit circadian variation, very little is known about their possible influence on excitation–contraction (E-C) coupling. We set out to determine whether the time of day impacts E-C coupling and its regulation by sympathetic stimulation. Paradoxically, our data show that the Ca$^{2+}$ transient responsible for contraction is larger, and the response to isoproterenol is also significantly greater, during the day, when the rat is at rest. This results from an enhanced Ca$^{2+}$ content of the sarcoplasmic reticulum. The increased response of the Ca$^{2+}$ transient to isoproterenol during the daytime is correlated with a decrease in neuronal NO synthase (nNOS) activity. This heightened sensitivity to $\beta$-adrenergic stimulation during the resting period of the rat resulted in a reduced arrhythmic threshold to isoproterenol. In man, the early morning prevalence of sudden cardiac death resulting from ventricular arrhythmias may reflect a delay in switching from this heightened sensitivity to sympathetic stimulation during wakening. Pharmacological targeting of the nNOS signaling pathway may prove useful in the management of life-threatening arrhythmias.
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Materials and methods

1. Animals
Adult male Wistar rats (around 300g) were housed in environmentally controlled rooms (12 hour light/dark cycle) and randomly assigned to one of two separate rooms with opposing light cycles. The normal light cycle room had a light regime of lights on (Zeitgeber time of ZT0) at 6:00 AM and the reverse-light cycle with lights on at 6:00 PM. Animals were euthanized at 9:00 AM by cervical dislocation, equivalent to a Zeitgeber time of ZT3 for animals in the normal-light cycle or ZT15 in the reverse-light cycle room, which in the nocturnal rat corresponds to the resting period (ZT3) and the active period (ZT15) of the animal.

2. Enzymatic isolation of single ventricular myocytes
Animals were euthanized at the specified time points by a blow to the head followed by cervical dislocation. A thoracotomy was performed and the heart rapidly removed and placed in cold Ca\textsuperscript{2+}-free Tyrode solution. The heart was then cannulated at the aorta within 60 seconds and perfused at a constant perfusion rate of 10 mls/min with Ca\textsuperscript{2+}-free Tyrode, vigorously bubbled with 100% oxygen, for 6 minutes. The heart was then perfused for 5 minutes with an enzyme solution containing type I Collagenase (1 mg/ml) and type XIV Protease (0.67 mg/ml) and BSA fraction V (1.67 mg/ml) (Sigma) in Ca\textsuperscript{2+}-free Tyrode to digest the heart, followed by normal Tyrode containing 2mM CaCl\textsubscript{2} to remove and inactivate the enzymes. The single myocytes were released by gently shaking the coarsely chopped left ventricle in normal Tyrode. Induction of the Ca\textsuperscript{2+}-paradox during this Ca\textsuperscript{2+}-depletion phase is prevented by the presence of 1 mM MgCl\textsubscript{2} \textsuperscript{1}.

This method of isolation produces between 80-90% viable, Ca\textsuperscript{2+}-tolerant rod shaped myocytes. We have previously shown that these myocytes behave in a similar fashion to intact heart tissue with respect to; their response to; the Ca\textsuperscript{2+}-paradox \textsuperscript{1}, ischaemic reperfusion injury \textsuperscript{2} and ischaemic and pharmacological preconditioning \textsuperscript{3-5}. We have looked at activation of the stress kinase PKC\delta and \epsilon in isolated myocytes and have found that these are not significantly different to levels in ex-vivo freshly isolated heart tissue. The isolation process takes 30 minutes and experiments were carried out on the day of isolation and no significant variation was found between data obtained over the experimental period (< 8 hours).

3. Measurement of intracellular calcium and L-type Ca\textsuperscript{2+}-current current-voltage relationships
Our method for measurement of [Ca\textsuperscript{2+}]\textsubscript{i} has been described previously \textsuperscript{2, 5}. Myocytes were loaded with Fura-2 (5 \textmu mol/l) for 15 minutes and washed twice with normal Tyrode and left for 30 minutes prior to use. Myocytes were illuminated alternately with 340/380 nm using a monochromator and emitted light collected using a photomultiplier tube (Photon Technology International). Ca\textsuperscript{2+}-transients were recorded from myocytes field-stimulated at 1 Hz and SR Ca\textsuperscript{2+}-content was estimated by the rapid application of caffeine 20mM.

L-Type Ca\textsuperscript{2+} current density was recorded from single ventricular myocytes using conventional patch pipettes in the whole-cell configuration, with an Axopatch 200B patch clamp amplifier (Axon Instruments Inc., Foster City, California). Patch pipettes were filled with a solution containing (in mM): CsCl 150, adenosine triphosphate, disodium salt (Na\textsubscript{2}ATP) 3, MgCl\textsubscript{2} 5, 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) 10, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10, titrated to pH 7.2 with KOH, and had resistances of 4-6 M\textOmega. The myocyte was voltage clamped at a holding potential of -45 mV, to inactivate the fast Na\textsuperscript{+} current, and depolarizing pulses of 200 ms duration were applied to the myocyte to test potentials between -40 to +80mV in +10 mV steps at 0.5Hz. BAPTA was included to protect the cell against Ca\textsuperscript{2+}-overload injury during maximal stimulation with isoproterenol and to reduce any indirect actions of isoproterenol, through changes in [Ca\textsuperscript{2+}]\textsubscript{i}, on Ca\textsuperscript{2+}-current amplitude. However, the presence of BAPTA alters Ca\textsuperscript{2+}-channel inactivation kinetics, slowing Ca\textsuperscript{2+}-dependent inactivation \textsuperscript{6}, and as such analysis of channel kinetics was not performed in this study.
We measured the membrane capacitance ($C_m$) of the myocytes using the membrane test function of pCLAMP 9, to facilitate electronic compensation and to normalize the L-type Ca$^{2+}$-current data to current density. The average $C_m$ was not significantly different between time points, at 123.3 ± 5.1 pF ($n = 53$) in resting period and 116.8 ± 3.7 pF ($n = 52$) in active period myocytes. All experiments were conducted at 35 ± 1°C with the aid of a bath temperature controller and this, in addition to the presence of intracellular BAPTA, resulted in an enhanced Ca$^{2+}$-current density $^{19}$.

4. Determination of arrhythmias in single ventricular myocytes

Myocytes were field-stimulated at 1Hz and superfused with Tyrode containing isoproterenol for 5 minutes. The percentage of cells displaying arrhythmic activity, defined as extra systolic contractions independent of the electrical stimulation was determined from a field of myocytes (8-15 cells) at the end of the 5 minutes superfusion. Under basal conditions, with the myocytes superfused with normal Tyrode, <2% of cells were found to have arrhythmic activity and this was not significantly different between resting or active phase myocytes. In a separate set of experiments, action potentials were recorded from single myocytes stimulated at 1Hz and superfused with Tyrode containing 100nM isoproterenol, where the electrode solution contained 150 mmol/l KCl and 0 BAPTA. In these experiments, we only ever recorded delayed after-depolarizations ($n = 10$ resting period and $n = 10$ active period myocytes), which lead to extra systoles and spontaneous contractions (Online Figure 1).

5. Measurement of left ventricular developed pressure (LVDP)

Hearts were removed from the animal euthanized during the resting period or active period and immersed in cold Tyrode solution. The heart was then cannulated at the aorta and perfused using a constant flow Langendorff apparatus (10 ml min$^{-1}$, GilsonMinipuls 3 peristaltic pump) with normal Tyrode bubbled vigorously with 100% oxygen, and the temperature was maintained at 37°C. The left ventricular effluent was drained via a catheter placed at the left ventricular apex and left ventricular pressure (LVP) was monitored with a fluid-filled latex balloon using an MTL0380 pressure transducer (ADInstruments Ltd, UK), inflated to an end-diastolic pressure of 6-10 mmHg. The pressure transducer was calibrated using a mercury filled manometer. Perfusion pressure was monitored with a second pressure transducer in series with the aortic cannula. The right atrium was removed and the heart paced using a bipolar electrode inserted into the right ventricle via the right ventricular outflow tract.

6. Drugs and experimental solutions

Tyrode solution contained (mmol/l): NaCl 135, KCl 5, NaH$_2$PO$_4$ 0.33, Na-pyruvate 5, glucose 10, MgCl$_2$ 1, CaCl$_2$ 2, Heps 10, titrated to pH 7.4 with NaOH. CaCl$_2$ was omitted from the Ca$^{2+}$-free Tyrode. Fura-2 (5 mmol/l) (Molecular Probes Inc.) was dissolved in DMSO containing 5% pluronic acid. Isoproterenol (ISO) was dissolved in ethanol (10 mmol/l), the nNOS specific inhibitor N-[(4S)-4-amino-5-[(2-aminoethyl)amino]pentyl]-N’-nitroguanidine tris(trifluoroacetate) (4-AAPNT) was dissolved in DMSO (2 mmol/l), prior to dilution into Tyrode solution and N-$\omega$-Nitro-L-arginine (L-NNA) was added directly to Tyrode solution.

7. Determination of mRNA expression of EC-coupling genes

Left ventricular free wall was dissected from hearts excised from rats euthanized during the resting or active phase, and snap frozen in liquid nitrogen. Additional experiments were conducted to determine RNA levels in single myocytes isolated from the left ventricle during the resting and active phase. Left ventricular tissue or single cells were homogenised and total RNA was extracted using an RNeasy plus mini kit, which includes a genomic DNA removal column and subsequent spin column technology, and a RNase-free DNase set was used as an additional DNase step (Qiagen). High quality RNA was reverse transcribed into cDNA on a PTC-200 Peltier thermal cycler using a High capacity RNA to cDNA kit, which incorporates both random hexamer and oligo (dT) primers in a mix of 10:1, respectively (Applied Biosystems). Non-template and non-enzyme controls were also performed at the reverse transcription stage to test for DNA contamination.
mRNA expression was analysed and quantified using fluorescent probe based Taqman gene expression assays (For probe details see supplemental table 1), in combination with the endogenous control, β-actin (ACTB, Applied Biosystems), to which all mRNA levels were normalised. PCR reactions were run in triplicate each with 50ng of starting cDNA template in volumes of 20µl using an ABI-Prism 7900HT sequence detector real-time PCR machine (Applied Biosystems) and the PCR amplification was performed to the specifications of the manufacturer’s protocol. The resulting mRNA expression data was analysed using the ΔΔCt method or the Pfaffl method, where necessary. All Taqman probes used in this investigation produced linear standard curves with PCR efficiencies of between 80-110%. Gene expression levels were normalized (ΔCt) to the house keeping gene β-actin, which was not found to cycle in any of our samples with a CT value of 18.54 ± 0.02 in resting phase myocytes and 18.56 ± 0.03 in active phase myocytes (n= 48, 45).

For the Taqman probes that are capable of amplifying genomic DNA (see table 1), an additional DNase I (Sigma) treatment was applied to RNA prior to reverse transcription. Briefly, this protocol involved diluting approximately 1µg of each high quality RNA sample in nuclease-free water (8µL), to which both reaction buffer (1µl) and DNase I (1µl) are added. The samples were mixed and left at room temperature for 15 minutes, to allow the DNase time to successfully remove any contaminating DNA. After which, a “stop solution” containing 50mM EDTA was added to stop the reaction and then all the samples were heated to 70°C for 10 minutes on a Techgene thermal cycler.

7.1 ΔΔCT method of mRNA analysis
The comparative ΔΔCT method of analysing mRNA expression was employed when the PCR efficiencies of the gene of interest and endogenous control gene (β-actin) were equal. The formula used for the comparative ΔΔCT method (equation 1) is outlined below.

$$\Delta C_T = Mean\ C_T^{(gene\ of\ interest)} - Mean\ C_T^{(β-actin)}$$

$$\Delta\Delta C_T = \Delta C_T^{(ZT15)} - \Delta C_T^{(calibrator, ZT3)}$$

Fold change = $$2^{-\Delta\Delta C_T}$$  
(Equation 1)

7.2 Pfaffl method of mRNA analysis
When the efficiencies of the gene of interest differed > ± 5% of the endogenous control gene (β-actin at 95%), the comparative ΔΔC_T method of mRNA analysis was not used, as it assumes an exponential PCR amplification. Therefore, the Pfaffl correction (equation 2) was applied, which takes into account the difference in efficiencies during analysis.

$$\Delta C_P^{(gene\ of\ interest)} = Mean\ CT\ of\ gene^{(Calibrator\ sample)} - Mean\ CT\ of\ gene^{(Unknown\ sample)}$$

$$\Delta C_P^{(control\ gene)} = Mean\ CT\ of\ gene^{(Calibrator\ sample)} - Mean\ CT\ of\ gene^{(Unknown\ sample)}$$

Fold change (Ratio) = $$\frac{(E_{Gi})\Delta C_P^{gene\ of\ interest}}{(E_{Gc})\Delta C_P^{control\ gene}}$$  
(Equation 2)

Where;

$$E_{Gi}$$ = Efficiency of gene of interest

$$E_{Gc}$$ = Efficiency of control gene (β-actin)

In this study, the calibrator refers to resting phase samples and unknown refers to active phase samples.
8. Data acquisition and analysis
Electrophysiological data were recorded directly to hard disk using pClamp 9.2 software. Statistical significance was calculated using a two-way ANOVA as appropriate followed by a Bonferroni post hoc test for significance or student-t test as appropriate. Data are presented as mean ± S.E.M. For number of experiments, we have given numbers of cells and hearts within each experiment. For arrhythmia data an experiment refers to a field of 8-15 cells and number of hearts. Experiments were conducted at 35°C.

9. Circadian cycling of CLOCK and Per2 in Wistar rat left ventricular tissue and isolated left ventricular myocytes
Exposure of cultured adult rat cardiomyocytes to foetal calf serum (50%) for 2 hours has been shown to induce circadian clock gene oscillation, 12. As our isolation process involves exposure of the heart to BSA (<1%) albeit for only 5 minutes during the enzyme digestion, we first confirmed that the profile of expression of two circadian clock genes, Per-2 and CLOCK in heart tissue, was similar to previous reports in the literature. We then compared the profile of Per-2 and CLOCK in isolated left ventricular myocytes and snap frozen ventricular tissue from the left ventricular free-wall of rats during the resting and active phases.

Online Figure IIA shows the diurnal pattern of expression of CLOCK and Per-2 is similar to previous reports in cardiac tissue 12, 13. Online Figure IIB shows that CLOCK and Per2 mRNA expression profiles of both circadian genes were not significantly different between isolated myocytes and intact ventricular tissue, suggesting that the isolation process has not adversely altered the circadian clock.
References

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**Online Table I**

Taqman gene expression probe based assays used in this investigation

Taqman gene expression probes used to assess the mRNA levels of circadian clock and EC-coupling genes. The “m1” probes react with cDNA at a junction between two exons and therefore, no contaminating genomic DNA (gDNA) is amplified. The “s1” probes however, span a single exon and as a result are capable of amplifying any contaminating gDNA. In these cases, the cDNA samples were treated within an additional DNase step prior to reverse transcription.

**Online Figure I**

**Action potentials, early after-depolarizations and extra systoles induced by isoproterenol**

Example of action potentials recorded from resting period myocytes showing the presence of delayed after-depolarization (left) and extra-systole (left) during superfusion with isoproterenol (100nmol/l).
Online Figure II

A. Diurnal variation in CLOCK (left) and Per2 (right) from Wistar rat hearts (n = 6).

B. Bar chart showing QRT-PCR of CLOCK and Per2 mRNA expression as fold-changes in left ventricular free wall (left) and isolated ventricular myocytes (right) obtained from Wistar rats during the resting period (open) and active period (grey). Both CLOCK and Per2 mRNA were normalised to b-actin mRNA. Fold-change of CLOCK and Per2 mRNA were calculated using the Pfaffl method relative to resting period tissue (the control). Mean ± S.E.M. n = 6; *p < 0.05, ***p < 0.001, unpaired students t test performed on ∆CT values.