Mechanisms Underlying Rate-Dependent Remodeling of Transient Outward Potassium Current in Canine Ventricular Myocytes

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Abstract—Transient outward K⁺ current (Iₒ) downregulation following sustained tachycardia in vivo is usually attributed to tachycardiomypathy. This study assessed potential direct rate regulation of cardiac Iₒ and underlying mechanisms. Cultured adult canine left ventricular cardiomyocytes (37°C) were paced continuously at 1 or 3 Hz for 24 hours. Iₒ was recorded with whole-cell patch clamp. The 3-Hz pacing reduced Iₒ by 44% (P<0.01). Kv4.3 mRNA and protein expression were significantly reduced (by ~30% and ~40%, respectively) in 3-Hz paced cells relative to 1-Hz cells, but KChIP2 expression was unchanged. Prevention of Ca²⁺ loading with nimodipine or calmodulin inhibition with W-7, A-7, or W-13 eliminated 3-Hz pacing-induced Iₒ downregulation, whereas downregulation was preserved in the presence of valsartan. Inhibition of Ca²⁺/calmodulin-dependent protein kinase (CaMKII) with KN93, or calcineurin with cyclosporin A, also prevented Iₒ downregulation. CaMKII-mediated phospholamban phosphorylation at threonine 17 was increased in 3-Hz paced cells, compatible with enhanced CaMKII activity, with functional significance suggested by acceleration of the Ca²⁺ transient decay time constant (Indo 1-acetoxyethyl ester microfluorescence). Total phospholamban expression was unchanged, as was expression of Na⁺/Ca²⁺ exchange and sarcoplasmic reticulum Ca²⁺-ATPase proteins. Nuclear localization of the calcineurin-regulated nuclear factor of activated T cells (NFAT)c3 was increased in 3-Hz paced cells compared to 1-Hz (immunohistochemistry, immunoblot). INCA-6 inhibition of NFAT prevented Iₒ reduction in 3-Hz paced cells. Calcineurin activity increased after 6 hours of 3-Hz pacing. CaMKII inhibition prevented calcineurin activation and NFATc3 nuclear translocation with 3-Hz pacing. We conclude that tachycardia downregulates Iₒ expression, with the Ca²⁺/calmodulin-dependent CaMKII and calcineurin/NFAT systems playing key Ca²⁺-sensing and signal-transducing roles in rate-dependent Iₒ control. (Circ Res. 2008;103:0-0.)

Key Words: potassium channels ■ calcium ■ calmodulin ■ remodeling ■ arrhythmias

Sudden cardiac death caused by ventricular tachycardia or fibrillation is an important contributor to mortality in congestive heart failure (CHF) patients. Rapid heart-rhythms can impair cardiac function and patients with “tachycardiomypathy” are at risk of sudden cardiac death. Chronic ventricular tachypacing in experimental animals produces a dilated cardiomyopathy that mimics clinical tachycardiomypathy and is often used as an experimental model to study CHF-related cardiac remodeling. Changes in cardiac ion channel transport are important components of this remodeling, and extensive evidence suggests that these ion transport changes are crucial contributors to the pathogenesis of CHF-related ventricular tachyarrhythmias and sudden death. Among the most ubiquitous changes are alterations in the transient outward K⁺ current (Iₒ), which play potentially important roles in repolarization abnormalities, cardiac dysfunction, and arrhythmogenesis. Although CHF itself can cause Iₒ downregulation, the possibility that rapid cardiomyocyte rate per se can alter Iₒ function has not been examined. This possibility cannot be examined directly in vivo, because sustained tachycardia causes a CHF syndrome, with major attendant hemodynamic, neurohumoral, and autonomic nervous system alterations, making it impossible to discern the role of heart rate per se. In the present study, we used a model of paced adult canine ventricular cardiomyocytes to determine: (1) whether increases in firing rate alter Iₒ; and if so, (2) the signaling systems involved.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

In Vitro Cellular-Pacing Model
Animal care procedures followed NIH guidelines. Adult male mongrel dogs (20 to 37 kg, n=114) were anesthetized with pentobarbital (30 mg/kg IV). Epicardial cardiomyocytes were isolated as previously described. After isolation, cells were kept in medium 199
and centrifuged (500 rpm, 1 minute, 25°C). Cell pellets were resuspended and plated on laminin-coated glass coverslips (for electrophysiology or immunohistochemistry) or 4-well rectangular petri dishes (Western blot or real-time RT-PCR). After a 4-hour preincubation, cells were divided into groups for parallel study in each experimental protocol and were electrically paced with 5-ms pulses at 1 or 3 Hz (1- and 3-Hz paced cells, respectively) for 24 hours, unless otherwise indicated. After pacing, cells were kept in a high-K⁺ storage solution at 4°C for electrophysiological studies or were fast-frozen at −80°C for biochemical studies.

**Electrophysiology**

$I_\text{Na}$ was recorded at 36±0.5°C (for details see the online data supplement, expanded Materials and Methods section). Currents are expressed as current densities (normalized to cell capacitance). Resting potentials, compensated series resistances, cell capacitances, and cell dimensions were similar for 1- and 3-Hz groups (online data supplement, Table I). Correction for liquid junction potentials (which averaged ~10 mV) was applied only for resting potential and reversal potential values.

**Real-Time PCR**

Total RNA was extracted from 1- and 3-Hz paced cells with TRIzol. Real-time reverse transcription (RT)–quantitative PCR was performed with TaqMan assays for Kv4.3, Kv1.4, and KChIP2 with 18S ribosomal RNA as the internal control.

**Immunoblotting**

Membrane protein fractions were isolated from 1- and 3-Hz paced cells incubated without (CTL) or with KN93 or KN92. Proteins were resuspended and plated on laminin-coated glass coverslips (for Western blot or real-time RT-PCR). After a 4-hour preincubation, cells were divided into groups for parallel study in petri dishes (Western blot or real-time RT-PCR). After a 4-hour preincubation, cells were divided into groups for parallel study in petri dishes (Western blot or real-time RT-PCR). After 24-hour pacing, cells were washed with culture medium, then fixed with 2% paraformaldehyde and washed 3 times (5 minutes each) with PBS. After blocking and permeabilization (2% normal donkey serum and normal goat serum, along with 0.2% Triton X-100), cells were incubated overnight at 4°C with primary antibodies against GAPDH (internal control for protein loading), α-actin (internal control for protein loading), ribosomal RNA as the internal control.

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**Confocal Microscopy**

After 24-hour pacing, cells were washed with culture medium, then fixed with 2% paraformaldehyde and washed 3 times (5 minutes each) with PBS. After blocking and permeabilization (2% normal donkey serum and normal goat serum, along with 0.2% Triton X-100 for 1 hour), cells were incubated overnight at 4°C with primary antibodies for NFATc3 (1:100, mouse monoclonal) and NFATc4 (1:100, rabbit polyclonal) in PBS containing 1% normal goat serum and 0.05% Triton, followed by 3 washes and secondary antibody (donkey anti-mouse Alexa-488 and goat anti-rabbit Alexa-488) incubation. Cells were then treated with RNaseA (100 μg/mL) and incubated with ToPro3 (1 μmol/L, for nuclear contour definition). Confocal microscopy was performed with a Zeiss LSM-510 system. Images were deconvolved using measured point spread functions. Nuclear and cytosolic NFATc3 and NFATc4 staining densities were determined as the sum of the pixels within each region normalized to region area. Measurements were repeated in 5 Z-stacks showing the maximum nuclear area in each cell.

**Calcineurin Activity**

Paced cell samples were collected after 6 hours of 1- or 3-Hz CTL, 3-Hz+KN93, or 3-Hz+KN92 (culture media containing 1 μmol/L KN93 or KN92) pacing based on preliminary studies showing peak activity after 6-hour 3-Hz pacing. Calcineurin activity was assessed with the Calcineurin Cellular Activity Assay Kit (Calbiochem).

**Data Acquisition and Analysis**

Clampfit 6.0 (Axon) and GraphPad Prism 3.0 were used for data analysis; curve fitting was performed with nonlinear least-square algorithms. Group comparisons were performed with paired or unpaired Student t tests or repeated-measures ANOVA with Bonferroni-corrected t tests or Dunnett’s tests. A 2-tailed P<0.05 indicated statistical significance; group data are expressed as means±SEM.

**Results**

Rapid Rates Downregulate $I_\text{Na}$ and Kv4.3

Representative $I_\text{Na}$ recordings from cells paced at 1 Hz (to mimic normal resting heart rates) and 3 Hz (to mimic tachycardia) are shown in Figure 1A. Cells paced at 3 Hz showed smaller $I_\text{Na}$ with otherwise similar morphology versus 1-Hz paced cells and had significantly smaller $I_\text{Na}$ densities over a wide range of voltages, with an ~45% reduction (Figure 1B). $I_\text{Na}$ inactivation kinetics were well fitted by biexponential relations, with inactivation time constants not different between 1- and 3-Hz cells (Figure 1C). The voltage dependence of $I_\text{Na}$ inactivation was studied with a 2-pulse protocol (Figure 1D, inset). Boltzmann relation fits showed no differences between 1- and 3-Hz: $V_{1/2}$ values and slope factors averaged −39.9±2.8 and −3.8±0.1 mV, respectively, in 1-Hz (n=6) and −39.8±1.8 and −4.1±0.1 mV in 3-Hz cells (n=7, P=NS versus 1-Hz). $I_\text{Na}$ activation voltage dependence was assessed based on the relation $I_\text{Na}(V)=I_{\text{Na}}(V/V_r)(G_r/G_{\text{max}})$, where $I_\text{Na}$ and $G_r$ are current and conductance at voltage $V$, $I_{\text{Na}}$ and $G_{\text{max}}$ are maximum current and conductance; and $V_r$ is the reversal potential. $V_r$ was determined by analyzing tail currents after 2.2-ms depolarizations to +50 mV and averaged −75.9±0.5 mV in 1-Hz (n=5) and −75.4±2.3 mV in 3-Hz cells (n=5; P=NS). $I_\text{Na}$ activation $V_{1/2}$ averaged 8.9±0.6 mV in 1-Hz (n=9) and 9.7±1.5 mV in 3-Hz cells (n=8, P=NS). $I_\text{Na}$ reactivation (2-pulse protocol, Figure 1E) was well fitted by biexponential relations. Recovery time constants (τ) averaged 30±2 ms (τ fast) and 130±21 ms (τ slow) in 1-Hz (n=7) and 29±3 (τ fast) and 149±12 ms (τ slow) in 3-Hz cells (n=8, P=NS versus 1-Hz). $I_\text{Na}$ frequency dependence based on steady-state currents at 0.1, 0.5, 1, 2, and 5 Hz (100-ms pulses from −80 to +50 mV) was not different between 1- and 3-Hz cells (supplemental Figure I). To assess $I_\text{Na}$ downregulation in vivo, we tachypaced 4 dogs at 240 bpm for 24 hours and compared $I_\text{Na}$ on freshly isolated cardiomyocytes from tachypaced and CTL dogs. The results (supplemental Figure II) show significant decreases in $I_\text{Na}$ consistent with in vitro observations.

To address the potential mechanisms underlying $I_\text{Na}$ downregulation, we first assessed mRNA and protein expression of potential underlying subunits: Kv4.3, Kv1.4, and KChIP2. The 3-Hz pacing significantly downregulated Kv4.3 mRNA (Figure 2A). Kv1.4 and KChIP2 mRNA expression was unaffected by 3-Hz pacing. Figure 2B shows examples of Kv4.3, KChIP2, and GAPDH immunoblots (top) and overall mean±SEM protein expression (bottom). Consistent with mRNA results, 3-Hz pacing downregulated Kv4.3 protein expression by ~40%, whereas KChIP2 protein expression was unchanged.
These data indicate that rapid firing rates reduce $I_{\text{to}}$ density through downregulation of Kv4.3 mRNA and protein. We next determined whether $I_{\text{to}}$ downregulation requires cardiomyocyte mechanical activity and associated metabolic demands or whether electric activity is sufficient for downregulation. Blebbistatin (5 $\mu$mol/L), an excitation–contraction uncoupler with minimal direct electrophysiological actions,9 was added to the culture medium during 24-hour pacing at 1 and 3 Hz. Ca$^{2+}$ transient activity in the absence of cell shortening confirmed cell capture during electromechanical uncoupling, as previously described.10 Supplemental Figure IIIA shows $I_{\text{to}}$ recordings from cells studied in parallel with 1- and 3-Hz pacing, with and without blebbistatin, which failed to prevent rate-induced $I_{\text{to}}$ downregulation (supplemental Figure IIIB). We then addressed the possibility that the results of 3-Hz stimulation could be attributable to direct effects of larger total durations of electric be attributable to direct effects of larger total durations of electrical field stimulation. In parallel experiments, we subjected cells to 1- and 3-Hz pacing with 3-ms stimuli, as well as to 1-Hz stimulation with 9-ms stimuli (to provide the same total field stimulation duration as 3-Hz 3-ms stimuli), keeping stimulus intensities constant. As shown in supplemental Figure IV, 1-Hz stimulation with 9-ms pulses failed to reproduce the effects of 3-Hz 3-ms stimuli. We next determined whether downregulation of $I_{\text{to}}$ requires angiotensin II receptor stimulation. Cells were subjected to 24 hours of 1- or 3-Hz pacing in the presence of the type-1 angiotensin II receptor antagonist valsartan (1 $\mu$mol/L). Valsartan failed to alter normalized results for Kv4.3 (n=8 cells per group). **P<0.001 vs 1-Hz. TP indicates test potential. C, Mean±SEM, $I_{\text{to}}$ inactivation $\tau$ values (n=8 cells per group). D, Mean±SEM voltage dependence of $I_{\text{to}}$ inactivation and activation. E, $I_{\text{to}}$ reactivation time course evaluated by the ratio of current ($I_{2}$) during a 100-ms test pulse ($P_{2}$) (HP=−80 mV, step to +50 mV at 0.07 Hz) to current ($I_{1}$) during a conditioning pulse ($P_{1}$) identical to $P_{2}$ with varying $P_{1}$ to $P_{2}$ interval. Data are means±SEM; curves are biexponential fits.

Figure 2. A, Mean±SEM normalized results for Kv4.3 (n=12 per group), KChIP2 (n=12 per group), and Kv1.4 (n=7 per group) real-time PCR obtained with 1- and 3-Hz paced cells. **P<0.001, 3- vs 1-Hz. B, Top, Examples of Kv4.3, KChIP2, and GAPDH (performed on the same samples as in the lanes above) Western blots from 1- and 3-Hz paced cells. Kv4.3 and KChIP2 bands were seen at the expected molecular masses (~70 and ~32 kDa, respectively). Bottom, Mean±SEM. Kv4.3 and KChIP2 expression levels relative to 1-Hz cell values (n=6 per group). **P<0.01 vs 1 Hz.
Role of Ca\(^{2+}\) Entry and Calmodulin

Intracellular [Ca\(^{2+}\)] and Ca\(^{2+}\) binding to calmodulin are dynamic, changing on a beat-to-beat basis in cardiomyocytes.\(^{11}\) If [Ca\(^{2+}\)], changes are important in mediating the \(I_{\text{Ca}}\) frequency response, suppressing activation-related Ca\(^{2+}\) entry through \(I_{\text{Ca}}\) should prevent rate-related \(I_{\text{Ca}}\) downregulation. Figure 3A and B show \(I_{\text{Ca}}\) recorded from cells cultured during 1- or 3-Hz pacing, in the presence of nimodipine (0.5 \(\mu\)mol/L), which decreased \(I_{\text{Ca}}\) by \(\approx 75\%\); supplemental Figure VI) or matching vehicle (CTL). The \(I_{\text{Ca}}\)-suppressant effect of 3-Hz pacing was eliminated by \(I_{\text{Ca}}\) blockade (Figure 3C). We then incubated cells with W-7 (1 \(\mu\)mol/L, to inhibit calmodulin) or vehicle and repeated these studies with 2 other calmodulin antagonists, A-7 (at 1 and 5 \(\mu\)mol/L) and W-13 (40 \(\mu\)mol/L), along with its inactive analog W-12 (40 \(\mu\)mol/L), because of potential concerns about the efficacy and specificity of W-7. Calmodulin inhibition prevented \(I_{\text{Ca}}\) downregulation in 3-Hz cells (Figure 3D and supplemental Figure VII). Thus, Ca\(^{2+}\)-dependent calmodulin function is implicated in rate-dependent \(I_{\text{Ca}}\) downregulation.

Role of CaMKII

High-frequency activation of Ca\(^{2+}\) transients increases CaMKII activity.\(^{12}\) To determine whether CaMKII activity is increased by 3-Hz pacing, we determined CaMKII-mediated Thr17 phosphorylation of PLB in cells cultured with vehicle (CTL), KN93 (1 \(\mu\)mol/L, a CaMKII inhibitor), or equivalent concentrations of the inactive analog KN92; preliminary experiments showed that 1 \(\mu\)mol/L KN93 had no effect on \(I_{\text{Ca}}\) (supplemental Figure VIII). CaMKII-phosphorylated PLB expression was significantly increased in 3-Hz paced cells (Figure 4A and B; for expanded Western blots see, supplemental Figure IXA), although total PLB expression was unaltered. For cells paced at 1 or 3 Hz in the presence of KN93 to inhibit CaMKII activity, there were no differences in Thr17 PLB phosphorylation (Figure 4C and 4D). Cells paced at 3 Hz in the presence of KN92 showed increased CaMKII PLB phosphorylation similar to CTLs (Figure 4E and 4F).

To obtain functional evidence for CaMKII activation, we studied the potential effect of CaMKII hyperphosphorylation of PLB, which should enhance the rate of removal of cytosolic Ca\(^{2+}\) via sarcoplasmic reticulum Ca\(^{2+}\) uptake (by removing PLB inhibition of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase function). Ca\(^{2+}\) transients were recorded with Indo 1-acetoxyethyl ester as previously described.\(^{13}\) Ca\(^{2+}\) transients recorded at a 1000-ms cycle length from 1- and 3-Hz paced cells are shown in Figure 4G. The Ca\(^{2+}\) transient decay time constant was significantly decreased in 3-Hz paced cells (Figure 4H; time constants averaged 345±35 ms [n=6] in 3-Hz cells versus 454±30 ms in 1-Hz cells [n=8];\(^{13}\) differences in Ca\(^{2+}\) transient decay time could also be attributable to alterations in other Ca\(^{2+}\)-handling proteins. The expression of other important Ca\(^{2+}\)-handling proteins was assessed (supplemental Figure X) and showed no effect of 3-Hz pacing.

To determine whether increased CaMKII activity contributes to the \(I_{\text{Ca}}\)-suppressing effect of 3-Hz pacing, \(I_{\text{Ca}}\) was recorded from cells exposed to vehicle (CTL), KN93 (1 \(\mu\)mol/L), or KN92 (1 \(\mu\)mol/L) during 24-hour 1- or 3-Hz pacing. Figure 5A and B show original recordings of \(I_{\text{Ca}}\) on depolarization to +40 mV from 1- and 3-Hz paced cells. CaMKII inhibition by KN93 prevented 3-Hz pacing-induced \(I_{\text{Ca}}\) reduction but had no effect on \(I_{\text{Ca}}\) in 1-Hz paced cells (Figure 5C). KN92 had no protective effect on \(I_{\text{Ca}}\) downregulation. We then studied the effects of CaMKII inhibition on Kv4.3 and KChIP2 protein expression (Figure 5D; expanded Western blots in supplemental Figure IXB). Whereas, in the presence of CaMKII inhibition with KN93, Kv4.3 protein expression was not reduced in 3-Hz cells, 3-Hz cells incubated in KN92 continued to show significant Kv4.3 downregulation. KChIP2 expression was unaltered in the
presence of KN93, excluding nonspecific effects on protein expression.

**Role of Calcineurin/NFAT System**

Ca\(^{2+}\)/calmodulin also activates calcineurin, a protein phosphatase that alters gene expression by dephosphorylating the transcription factor NFAT. We first compared calcineurin activity in 1- and 3-Hz paced cells at 6 hours after pacing initiation. As shown in Figure 6A, calcineurin activity was increased >2-fold in 3-Hz paced cells. Calcineurin protein expression was not altered after 6 hours of 3-Hz pacing (Figure 6B; expanded Western blots in supplemental Figure IXC), consistent with the notion that calcineurin was functionally activated by increased Ca\(^{2+}\) entry in 3-Hz cells. To test for the potential role of calcineurin in \(I_{\text{ca}}\) downregulation, \(I_{\text{ca}}\) changes were studied in 1- and 3-Hz paced cells incubated with cyclosporin A (1 \(\mu\)g/mL, \(\approx 0.8 \mu\)mol/L) or vehicle (CTL) during pacing (\(I_{\text{ca}}\) recordings shown in Figure 6C). Cyclosporin A prevented 3-Hz pacing-induced \(I_{\text{ca}}\)-reduction (Figure 6D), supporting the importance of calcineurin in \(I_{\text{ca}}\) downregulation.

To assess the potential role of the calcineurin downstream mediators NFATc3 and -c4, their cellular localization was studied by confocal microscopy. Deconvolved images of NFATc3 (red) and -c4 (green) staining are shown in Figure 7A. Figure 7B shows relative nuclear/cytosolic signal ratios. Examples of ToPro3 colocalization used to identify the nuclear region are shown in supplemental Figure XI. The NFATc3 nuclear/cytosolic staining ratio was significantly increased in 3-Hz cells, compatible with nuclear relocalization. To assess nuclear NFAT localization with an independent method, we performed immunoblots on purified nuclear extracts. The results confirmed increased nuclear NFATc3 localization in 3-Hz paced cells (supplemental Figure XII). We then applied a cell-permeable NFAT inhibitor, INCA-6, to study the functional importance of NFAT in the \(I_{\text{ca}}\) response.\(^{14}\) Cells were incubated with vehicle (CTL) or INCA-6 (5 \(\mu\)mol/L) during 24-hour pacing at 1 or 3 Hz. \(I_{\text{ca}}\) recordings at +40 mV are shown in Figure 7C, and mean data are shown in Figure 7D. INCA-6 prevented \(I_{\text{ca}}\) downregulation in 3-Hz cells, supporting the importance of NFAT as a mediator.

Our results point to the participation of both CaMKII and calcineurin systems as mediators of Ca\(^{2+}\)/calmodulin effects. Previous studies suggest potential crosstalk between CaMKII and calcineurin systems.\(^{15}\) We wondered whether crosstalk between these systems could be contributing to calcineurin-mediated effects in our model and assessed the effects of the CaMKII inhibitor KN93 or its inactive analog KN92 on calcineurin activation by 3-Hz pacing. Calcineurin activation was suppressed by CaMKII inhibition with KN93 (Figure 6A) but not by KN92, suggesting that intact CaMKII function is needed for 3-Hz pacing-induced enhancement of calcineurin function. Fur-
In our in vitro tachypaced cardiomyocyte model, accompanied by significant changes in biophysical properties. As shown in Figure 7B, KN93 (but not KN92) prevented NFATc3 nuclear translocation. A schematic summary of our findings is presented in Figure 8.

**Discussion**

In this study, we found that rapid cardiomyocyte firing decreases \( I_{\text{Ca}} \) density through downregulation of \( I_{\text{Ca}} \), \( \alpha \)-subunit (Kv4.3) gene and protein expression. Rate-dependent \( I_{\text{Ca}} \) downregulation is mediated by increased \( \text{Ca}^{2+} \)/calmodulin-activated CaMKII and calcineurin/NFAT signaling. Blockade of these pathways prevents rate-related \( I_{\text{Ca}} \) remodeling. A schematic summary of our findings is presented in Figure 8.

**Relation to Previous Studies of \( I_{\text{Ca}} \) Downregulation in Tachypaced Models**

Ventricular tachypacing is frequently used to create in vivo animal models of CHF. \( I_{\text{Ca}} \) downregulation is a consistent finding, generally with reductions in \( I_{\text{Ca}} \) density unaccompanied by significant changes in biophysical properties. In our in vitro tachypaced cardiomyocyte model, \( I_{\text{Ca}} \) density was similarly reduced with no change in voltage dependence or kinetic properties. As observed for in vivo tachypaced dog or rabbit models, Kv4.3 mRNA and protein were reduced, consistent with transcriptional downregulation.

Previous investigators have provided evidence suggesting that increased heart rate may be involved in cardiac hypertrophic signaling. The absence of changes in cellular capacitance and dimensions in 3-Hz paced cells make significant cellular hypertrophy unlikely in our model. Shortly after the onset of rapid electric stimulation (15 minutes), angiotensin II secretion and expression levels increase in cultured neonatal rat cardiomyocytes, returning to baseline after several hours. Incubation of epicardial ventricular myocytes with angiotensin II decreases \( I_{\text{Ca}} \) amplitude and changes its voltage-dependent and kinetic properties. Angiotensin receptor stimulation was not essential for \( I_{\text{Ca}} \) downregulation by 3-Hz pacing in our model, because significant downregulation continued to occur in the presence of AT1 receptor blockade with valsartan.

**Ca\(^{2+}\)/Calmodulin As a Rate Sensor**

Intracellular Ca\(^{2+}\) concentration changes provide key signaling messages in a variety of systems. The role of Ca\(^{2+}\) is particularly important in sensing alterations in the frequency and form of neuronal activity, with Ca\(^{2+}\)/calmodulin binding inducing CaMKII autophosphorylation and activation. Dynamic fluctuations in calmodulin-bound Ca\(^{2+}\) show both phasic components tracking intracellular Ca\(^{2+}\) concentration alterations and sustained changes that integrate Ca\(^{2+}\) concentrations over time. Inhibition of Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels suppresses long-term memory effects in vivo in dogs or tachycardia-induced decreases in Ca\(_{\text{1,2}}\) protein expression in HL-1 cells. The importance of Ca\(^{2+}\)/calmodulin sensing in rate-dependent \( I_{\text{Ca}} \) changes in our system was indicated by the ability of either Ca\(^{2+}\) channel blockade or calmodulin inhibition to prevent \( I_{\text{Ca}} \) downregulation.

**Role of CaMKII and Calcineurin Signaling**

Ca\(^{2+}\) response amplitude and duration are coupled to a variety of downstream regulatory systems, including transcriptional changes, for which NFAT is particularly important. NFAT is activated by calcineurin dephosphorylation of the NFAT regulatory domain, which is triggered by increased Ca\(^{2+}\)/calmodulin binding.

In vitro tachystimulation of neonatal rat cardiomyocytes or atrial tissue slices activates calcineurin/NFAT signaling. Calcineurin has been reported to alter \( I_{\text{Ca}} \) expression in a number of cardiac systems. Increased extracellular Ca\(^{2+}\)
concentration induces \( I_{\text{ca}} \) downregulation in rat ventricular cardiomyocytes because of reduced Kv4.2 mRNA expression, which is prevented by the calcineurin inhibitors FK506 or cyclosporin A.\(^{29}\) In mice, the transmural calcineurin-overexpressing/CaMKII-inhibited strains.\(^{34}\) We improved by CaMKII inhibitory drugs or in crossbred that their arrhythmias and left ventricular dysfunction are induced PLB phosphorylation. There is evidence for interac-

![Image](http://circres.ahajournals.org/)

**Figure 6.** A, Mean±SEM calcineurin activity in 1-, 3-, and 3-Hz paced cells cultured with KN93 or KN92 (n=15 per group for 1- and 3-Hz; n=9 per group for 3-Hz plus KN93 [3-Hz+93]; and n=8 per group for 3-Hz plus KN92 [3-Hz+92]). *P<0.05, **P<0.01 vs 1-Hz. B, Top, Examples of calcineurin (~61 kDa) and GAPDH Western blots from 1-, 3-, and 3-Hz cells with KN93 (3-Hz+93) or KN92 (3-Hz+92) treatment during 6-hour pacing. Bottom, Mean±SEM calcineurin protein expression after normal-

**Novel Findings and Potential Significance**

Although tachycardia-induced cardiomyopathy consistently causes \( I_{\text{ca}} \) downregulation in vivo,\(^{3-6} \) it is impossible in such a system to discriminate frequency-dependent phenomena from secondary changes attributable to altered hemodynamics, neurohormonal state, and the heart failure syndrome. Our study is, to our knowledge, the first to assess the effect of firing rate on \( I_{\text{ca}} \) in an adult cardiomyocyte system and to study underlying regulatory mechanisms. We have uncovered a complex system in which \( Ca^{2+} \) acts as a frequency sensor that couples via calmodulin to downstream signals that alter \( I_{\text{ca}} \) expression by changing the phosphorylation states of key proteins. Our results add to a growing body of evidence indicating that calcineurin/NFAT signaling acts to downregulate \( I_{\text{ca}} \) in a variety of physiological contexts, including tachycardia, \( Ca^{2+} \) loading caused by increased extracellular \( Ca^{2+} \),\(^{20} \) subendocardial tissues,\(^{30} \) and myocardial infarction.\(^{31} \) Like Rossow et al,\(^{30} \) we found increased calcineurin activity with unchanged calcineurin expression in a context of \( Ca^{2+} \) loading related \( I_{\text{ca}} \) regulation. In addition, we noted that CaMKII inhibition prevents calcineurin activity increases. Tachycardia shortens action potential duration, which would tend to reduce \( Ca^{2+} \) entry, as well as the time available for systole and cell contraction. Reductions in \( I_{\text{ca}} \) might offset this e

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CAMPKII inhibitor. Prior studies have shown CaMKII phosphorylation of calcineurin at very low \( Ca^{2+} \) concentrations that inhibit calcineurin function.\(^{15} \) It is conceivable that CaMKII may modulate calcineurin function by phosphoryl-

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**Novel Findings and Potential Significance**

Although tachycardia-induced cardiomyopathy consistently causes \( I_{\text{ca}} \) downregulation in vivo,\(^{3-6} \) it is impossible in such a system to discriminate frequency-dependent phenomena from secondary changes attributable to altered hemodynamics, neurohormonal state, and the heart failure syndrome. Our study is, to our knowledge, the first to assess the effect of firing rate on \( I_{\text{ca}} \) in an adult cardiomyocyte system and to study underlying regulatory mechanisms. We have uncovered a complex system in which \( Ca^{2+} \) acts as a frequency sensor that couples via calmodulin to downstream signals that alter \( I_{\text{ca}} \) expression by changing the phosphorylation states of key proteins. Our results add to a growing body of evidence indicating that calcineurin/NFAT signaling acts to downregulate \( I_{\text{ca}} \) in a variety of physiological contexts, including tachycardia, \( Ca^{2+} \) loading caused by increased extracellular \( Ca^{2+} \),\(^{20} \) subendocardial tissues,\(^{30} \) and myocardial infarction.\(^{31} \) Like Rossow et al,\(^{30} \) we found increased calcineurin activity with unchanged calcineurin expression in a context of \( Ca^{2+} \) loading related \( I_{\text{ca}} \) regulation. In addition, we noted that CaMKII inhibition prevents calcineurin activity increases. Tachycardia shortens action potential duration, which would trend to reduce \( Ca^{2+} \) entry, as well as the time available for systole and cell contraction. Reductions in \( I_{\text{ca}} \) might offset this effect by raising the plateau level and maintaining contraction strength. In pathological situations, however, \( I_{\text{ca}} \) downregul-

**Figure 6.** A, Mean±SEM calcineurin activity in 1-, 3-, and 3-Hz paced cells cultured with KN93 or KN92 (n=15 per group for 1- and 3-Hz; n=9 per group for 3-Hz plus KN93 [3-Hz+93]; and n=8 per group for 3-Hz plus KN92 [3-Hz+92]). *P<0.05, **P<0.01 vs 1-Hz. B, Top, Examples of calcineurin (~61 kDa) and GAPDH Western blots from 1-, 3-, and 3-Hz cells with KN93 (3-Hz+93) or KN92 (3-Hz+92) treatment during 6-hour pacing. Bottom, Mean±SEM calcineurin protein expression after normal-

**Novel Findings and Potential Significance**

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tion could promote arrhythmogenesis, particularly in contexts of abrupt rate slowing and reduced repolarization reserve.

Potential Limitations
There are well-recognized transmural differences in \(I_{\text{to}}\) density and properties.\(^{30,35}\) In addition, different regions of the heart vary in electrophysiological and ion current features.\(^{36}\) These could affect rate-dependent \(I_{\text{to}}\) regulation. We performed our studies in cells from the epicardium of canine left ventricles to prevent regional and transmural differences from adding uncontrolled variability to the results. A comprehensive study of the mechanisms of rate-dependent \(I_{\text{to}}\) regulation is beyond the scope of the present study, but the issue merits further investigation. The cell-permeable agents available for CaMKII inhibition, of which KN93 is the most widely used, have imperfect specificity, including potential \(I_{\text{CaL}}\)-blocking properties.\(^{37}\) For this reason, we were particularly careful to verify that the KN93 concentration we used does not block \(I_{\text{CaL}}\). However, we cannot totally exclude the possibility that actions of KN93 other than its CaMKII-inhibiting ability could have contributed to its effects in our system.

Tachycardia per se is clearly not the only factor that can alter \(I_{\text{to}}\) in ventricular tachypaced in vivo models. In addition to CHF-related neurohumoral activation and hemodynamic and metabolic changes, an altered sequence of ventricular activation can importantly alter cardiac repolarization by affecting \(I_{\text{to}}\). Yu et al have shown that 2-Hz ventricular pacing of dog hearts for 3 weeks slows \(I_{\text{to}}\) recovery from inactivation, positively shifts inactivation voltage dependence, and reduces conductance.\(^{38}\) Consistent with our findings, \(I_{\text{to}}\)-conductance decreases were associated with comparable reductions in mRNA expression; however, \(I_{\text{to}}\) kinetics and voltage dependence were not changed in our cells. The discrepancies may be attributable to differences in tachypacing rate (2 versus 3 Hz) and duration (24 hours versus 3 weeks), along with the absence of altered activation pattern-related factors in our in vitro model.

Figure 7. A, Immunolocalization of NFATc3 and NFATc4 in 1- and 3-Hz paced myocytes. B, Mean±SEM ratios of nuclear/cytosolic NFATc3 and NFATc4 fluorescence intensities expressed relative to 1-Hz values. The x axis provides the number of cells/experiments for each bar. NFATc3 nuclear/cytosolic ratios increased significantly with 3-Hz pacing relative to 1-Hz under CTL and KN92 conditions but not in the presence of KN93. No significant changes in NFATc4 ratios occurred. C, Examples of \(I_{\text{to}}\) recordings (same recording protocol as Figure 6) under CTL or INCA-6 (5 \(\mu\)mol/L) treatment conditions in 1- and 3-Hz paced cells. D, Mean±SEM. \(I_{\text{to}}\) densities at +40 mV for 1-Hz CTL and INCA-6–treated cells (n=10 cells per group), 3-Hz CTL, and INCA-6–treated cells (n=9 cells per group). **\(P<0.01\), 3-Hz INCA-6 vs 3-Hz CTL.
NFAT is generally associated with upregulation of gene expression in hypertrophic programs, but our results implicates NFAT in tachycardia-induced Kv4.3 downregulation. Recent work has established that NFAT may also selectively repress gene expression, with important consequences for lymphocyte, adipocyte, and activity-dependent skeletal muscle gene regulation.39

**Acknowledgments**

We thank Chantal St-Cyr, Chantal Maltais, and Nathalie L’Heureux for technical assistance and France Thériault for secretarial help with the manuscript.

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**Disclosures**

None.

**References**


Mechanisms Underlying Rate-Dependent Remodeling of Transient Outward Potassium Current in Canine Ventricular Myocytes
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Materials and Methods

In vitro Cellular Pacing Model

Cell Isolation. All animal care and handling procedures followed the Animal Care Guidelines of the National Institutes of Health and were approved by the animal research ethics committee of the Montreal Heart Institute. Male adult mongrel dogs (20–37 kg) were anesthetized with pentobarbital (30 mg/kg IV) under artificial ventilation. Left lateral thoracotomy was performed, and hearts were quickly excised and immersed in oxygenated Tyrode’s solution at room temperature. The transmural free wall (~30 × 50 mm) of the anterior left ventricle, which contains coronary artery branches from the left circumflex coronary artery, was quickly dissected and the artery was cannulated. Cell isolation was performed by perfusion with Tyrode’s solution containing collagenase (120 U mL⁻¹, Worthington, type II) as previously described.¹ Cells from the epicardial (Epi) surface (1~1.5 mm thick) were taken after digestion and were kept in culture medium for further studies.

Cell Culture and Pacing. All procedures for cell culture and pacing were performed under aseptic conditions. After cell isolation, the cell suspension was centrifuged at 500 rpm for 1 minute at 4°C and cell pellets were re-suspended in fresh culture medium. Culture medium contained Medium 199 (GIBCO, Invitrogen Corp., with Earle’s salts, L-glutamine and 2,200 mg/L sodium bicarbonate), Na-penicillin G (100-U/mL) and streptomycin sulfate (100-μg/L), and was supplemented with Insulin-Transferrin-Selenium-X (GIBCO, Invitrogen Corp., containing 0.01-mg/mL insulin, 5.5-μg/mL transferrin, 6.7-ng/mL sodium selenite and 2-μg/mL ethanolamine). Cells were plated at ~1×10⁴ cells/cm² on circular (18-mm diameter) or rectangular (24x55 mm) glass coverslips or 4-well rectangular petri dishes (24x67 mm/well,
Nalge Nunc International) pre-coated with laminin (15-µg/mL). Myocytes were maintained in the culture media at 37°C in a humidified, 5% CO2-enriched environment in the incubator. After 4 hours, any dead or unattached myocytes were washed off gently with fresh media to leave a homogeneous layer of rod-shaped cells attached to the coverslips or the 4-well petri dish. Cells were divided into 2 groups electrically paced at 1 Hz or 3 Hz respectively for 24 hours in the incubator. In some studies, cells were divided into different groups for both 1-Hz and 3-Hz paced cells, each including CTL (vehicle) or drug (nimodipine, W-7, A-7, W-13, W-12, KN93, KN92, cyclosporine-A or INCA-6 etc.). The electrical pacing system includes C-Pace100™ and C-Dish100™ units (IonOptix Corporation, Milton, MA, USA). The stimulation pulse duration was 5 milliseconds and the pulse voltage was 45±2 V. Evidence of capture was examined by microscopic observation at the beginning and before termination (after 24 hours pacing) of stimulation. Capture efficiency was ~100% for cells attached on laminin-coated coverslips or plates. After 24 hours, cells on coverslips were kept in the high-K+ storage solution at 4°C for electrophysiological studies. Cells directly on culture plate were scraped off with a cell scraper (SARSTEDT) and were centrifuged at 1000 rpm for 5 minutes at 4°C. Cell pellets were frozen and kept at -80°C immediately for further molecular biology studies.

**In vivo tachypacing**

Four mongrel dogs (22-26 kg) were subjected to *in vivo* right ventricular tachypacing (240 bpm) for 24 hours as previously described.⁵
Electrophysiology

Whole-cell patch-clamp technique (voltage-clamp mode) was applied for $I_{\text{to}}$ recordings at 36±0.5°C. Borosilicate-glass electrodes had tip resistances between 1.5 and 3.0 MΩ when filled. Cell capacitance and series resistance were compensated by ~80% to 90% to minimize the capacitive surge on the voltage recording. Leakage compensation was not used. Cell capacitances were not different between 1-Hz and 3-Hz paced cells (on-line Table 1). Currents are expressed in terms of density.

The standard Tyrode solution contained (in mmol/L) NaCl 136, KCl 5.4, MgCl$_2$ 1, CaCl$_2$ 1, NaH$_2$PO$_4$ 0.33, HEPES 5 and dextrose 10 (pH 7.35 with NaOH). The high-K$^+$ storage solution contained (in mmol/L) KCl 20, KH$_2$PO$_4$ 10, dextrose 10, mannitol 40, L-glutamic acid 70, β-OH-butyric acid 10, taurine 20, EGTA 10 and 0.1% BSA (pH 7.3 with KOH). The standard pipette solution used in most experiments contained (in mmol/L) K-aspartate 110, KCl 20, MgCl$_2$ 1, MgATP 5, GTP 0.1, HEPES 10, Na-phosphocreatine 5, EGTA 5 (for current recording) or 0.025 (for action potential (AP) recording), with pH adjusted to 7.3 with KOH.

For $I_{\text{to}}$ recordings, atropine (1-μmol/L) and CdCl$_2$ (200-μmol/L) were added in external solutions to eliminate muscarinic K$^+$-currents and to block Ca$^{2+}$-currents. Na$^+$-current contamination was avoided by using a holding potential (HP) of -50 mV or by substitution of equimolar Tris HCl for external NaCl. For L-type calcium current ($I_{\text{CaL}}$) studies, the external solution contained (in mmol/L) tetraethylammonium chloride (TEA-Cl) 136, CsCl 5.4, CaCl$_2$ 2, MgCl$_2$ 0.8, HEPES 10 and dextrose 10 (pH 7.4 with CsOH). Niflumic acid (50 μmol/L) was added to inhibit $I_{\text{Cl, Ca}}$. The pipette solution contained (mmol/L) CsCl 20, Cs-aspartate 110, MgCl$_2$ 1, MgATP 5, GTP 0.1, Na$_2$Phosphocreatine 5, EGTA 10 and HEPES 10 (pH 7.2 with CsOH).
Compounds

W-7, nimodipine, KN93 and KN92 were purchased from Calbiochem. Valsartan was kindly provided by Novartis, East Hanover, NJ. Cyclosporine A (CyA), W-13 and W-12 were purchased from Sigma. A-7 was purchased from e-BioMed GmbH. Drugs were prepared in stock solution with stock concentration being 1-mmol/L for W-7, nimodipine and valsartan, 5-mmol/L for A-7, W-13 and W-12, 25-mmol/L for KN93 and KN92, and 5-mg/mL for CyA. Individual control (CTL) vehicles were individual solvents to dilute the specific drug (DMSO for W-7, nimodipine, KN93 and KN92; H2O for valsartan, A-7, W-13 and W-12; ethanol for CyA).

Real-time PCR

Total RNA was extracted from 1-Hz and 3-Hz snap-frozen cell samples with homogenization in TRIzol Reagent (Invitrogen), chloroform extraction and isopropanol precipitation. Genomic DNA was eliminated by incubation in DNase I (0.1-U/μL, 37°C) for 30 minutes, followed by phenol-chloroform acid extraction and gel verification. RNA was quantified spectrophotometrically at a 260-nm wavelength and integrity was confirmed on a denaturing agarose gel. RNA samples were stored in DEPC H2O at -80°C. First-strand cDNA was synthesized by RT with 2 μg of RNA samples, random primers and MMLV reverse transcriptase (High Capacity cDNA Archive Kit, Applied Biosystems). Real-time PCR was conducted with Stratagene Mx3000P QPCR detection system and was performed with Taqman quantitative assay. Commercially purchased 18s rRNA (Applied Biosystems) was used as the internal control. Primers and probes for real-time PCR reaction are listed in on-line Table 2. Each sample was run in duplicate and PCR products were verified with gel electrophoresis. Kv4.3, Kv1.4 and KChIP2 were normalized to 18s rRNA; internal control data were obtained from the same samples at the
same time and there were no differences of 18s rRNA expression between 1-Hz and 3-Hz paced groups.

**Protein Extraction and Western blot**

**Protein extraction:** Membrane protein fractions were isolated with extraction buffer containing 25-mmol/L Tris-HCL (pH 7.4), 5-mmol/L EGTA, 5-mmol/L EDTA, 1-mmol/L Na$_3$VO$_4$, 0.5-mmol/L AEBSF, 1-mmol/L iodoacetamide and β-2-mercaptaethonal, 10-μg/mL aprotinine and leupeptin and 1-μg/mL pepstatin followed by homogenization. After centrifugation at 1000 rpm and 4°C for 5 minutes, the supernatant containing the cell membranes was centrifuged at 100,000 g for 1 hour. Membrane protein pellets were re-suspended in extraction buffer supplemented with 1% triton and stored at -80°C. For calcineurin detection, total cell lysates were extracted according to the manufacturer’s instruction as described in the Calcineurin activity assay kit.

**Cellular fractionation:** The nuclear isolation was done according to a customized version of a previously-described method.$^3$ Briefly, myocyte pellets were powdered under liquid nitrogen, resuspended in cold PBS and homogenized using a universal Polytron (8500rpm; 2x15s). The total extract was labelled as Fraction A. All the following steps were carried out on ice or at 4°C. Homogenates were centrifuged at 500 x g for 15 minutes and the supernatants, referred to as Fraction B, were diluted 1:1 with buffer A (10 mmol/L K-HEPES (pH 7.9), 1.5 mmol/L MgCl$_2$, 10 mmol/L KCl, 1 mmol/L DTT, 25 μg/mL leupeptin, 0.2 mmol/L Na$_3$VO$_4$), incubated for 10 minutes on ice and recentrifuged at a higher speed (2000 x g) for 15 minutes. The resulting supernatant was discarded. The pellet was considered to be crude nuclei (Fraction C), and was resuspended in buffer B (0.3 M K-HEPES, pH 7.9, 1.5 M KCl, 0.03 M MgCl$_2$, 25 μg/mL
leupeptin, 0.2 mmol/L Na₃VO₄), incubated on ice for 10 minutes, and centrifuged for 15 minutes at 2000 x g. The pellet, which is referred to as the enriched nuclear fraction (Fraction D), was resuspended in buffer C (20 mmol/L Na-HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L EDTA, 0.2 mmol/L EGTA, 0.5 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L DTT, 25 μg/mL leupeptin, 0.2 mmol/L Na₃VO₄) and snap frozen with liquid nitrogen and stored at -80°C. As shown in the image below, the respective fractions were loaded on SDS-PAGE gels, and the nuclear fraction showed enrichment of nucleoporin (NP62) and almost undetectable levels of the endoplasmic-reticulum marker GRP78. For experiments assessing nuclear localization of NFATc3 and c4, only fractions A, C and D were loaded on the membranes. Nuclear localization was assessed by quantifying total cell-extract (Fraction A) and nuclear-enriched (Fraction D) NFAT band-densities. Nucleoporin62 (NP62) was used as the loading control and the ratio of nuclear to total cell lysate level of NP62 was not different between 1-Hz and 3-Hz conditions.
Immunoblotting: Protein concentration was determined with the Bradford method. 20-40 μg of membrane protein samples, total cell lysates or total cell extract and nuclear extract from 1-Hz or 3-Hz cells was denatured with Laemmli buffer and was fractionated on 8% or 12% SDS-polyacrylamide gels, then proteins were transferred electrophoretically to Immobilon-P polyvinylidene fluoride (PVDF) membranes (Millipore) in 25-mmol/L Tris-base, 192-mmol/L glycine and 20%-ethanol at 0.3 A for 1 hour. Membranes were blocked in 1 X TBS-T with 5% non-fat dry milk for 1 hour and incubated with primary antibodies (goat anti-Kv4.3, 1:500, Santa Cruz; mouse anti-KChIP2, 1:5000, from Dr. Trimmer; rabbit anti-phospholamban [phosphorylated at threonine 17], 1:5000, Badrilla; mouse anti-phospholamban [total], 1 μg/mL, Affinity Bioreagent (ABR); mouse anti-calcineurin, 1:5000, BD Transduction; mouse anti-NCX, 1:2500, ABR; mouse anti-SERCA, 1:2500, ABR; mouse anti-RyR2, 1:1000, ABR; mouse anti-NFATc3, 1:200, Sigma; rabbit anti-NFATc4, 1:200, Sigma; rabbit anti-nucleoporin p62, 1:1000 BD Transduction; rabbit anti-GRP78 BiP, 1:1000, ABcam; mouse anti-annexin, 1:1000, BD Transduction) overnight at 4°C. After washing and re-blocking, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit, donkey anti-goat or goat anti-mouse IgG secondary antibody (1:10,000, Jackson Immunolabs or Santa Cruz). Antibody was detected with Western-Lightening Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences). Later, the same membranes were also probed with anti-GAPDH at room temperature for 2 hours in order to control for protein loading. Secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000, Santa Cruz).
Calcineurin-Activity Assay

Calcineurin-activity was assessed using the Calcineurin Cellular Activity Assay Kit (Calbiochem) according to the manufacturer’s instruction. Briefly, paced cell samples were collected at 6 hours during 1-Hz, 3-Hz CTL, 3-Hz+KN93 or 3-Hz+KN92 pacing: 0 hour (just before pacing). Samples were lysed in lysis buffer (25-mmol/L Tris-HCl (pH 7.5), 0.5-mmol/L DTT, 50-µmol/L EDTA, 50-µmol/L EGTA and 0.1% NP-40) with protease inhibitors. The cell lysates were incubated with RII phosphopeptide (750-µmol/L, the well known substrate for calcineurin) in assay buffer containing 100 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 6-mmol/L MgCl₂, 0.5-mmol/L CaCl₂, 0.5-mmol/L DTT, 0.025% NP-40 and 500-nmol/L Okadaic acid with or without 10-mmol/LM EGTA. After 30 minutes at 30°C, reactions were terminated by adding 100 µL GREENᵀᴹ reagent and fluorescence was measured at 620 nm using a 96-well plate reader (Tecan SPECTRA Rainbow, PAA Ltd.).

Confocal microscopy

After 24-hour pacing, the paced cells were washed with fresh culture medium, then fixed with 2%-paraformaldehyde (20 minutes, Sigma-Aldrich) and washed 3 times (5 minutes each) with phosphate buffered saline (PBS). Cells were blocked and permeabilized with 2% normal donkey serum (NDS, Jackson) and normal goat serum (NGS, Jackson) and 0.2% Triton X-100 (Sigma) for 1 hour. Cells were then incubated overnight at 4°C with primary antibodies for nuclear factor of activated thymocytes (NFAT) c3 (1:200, mouse monoclonal, Santa Cruz) and NFATc4 (1:100, rabbit polyclonal, Santa Cruz) in PBS containing 1%-NDS and NGS and 0.05%-Triton, followed by 3 washes and secondary antibody (donkey-anti-mouse Alexa-547 and goat-anti-rabbit Alexa-488, Jackson) incubation at room temperature for 1 hour. Cells were then treated with RNaseA
(100-µg/mL, Roche) for 25 minutes at 37°C followed by 3 washes, then incubated with ToPro3 (1-µmol/L, for nuclear labeling, emission at 661-nm, Invitrogen) for 45 minutes at room temperature. Confocal microscopy was performed with a Zeiss LSM-510 system. Control experiments omitting primary antibodies revealed absent or very low-level background staining. Images were deconvolved with Huygens Professional software (Scientific Volume Imaging) using measured point spread functions (PSFs). Measured PSFs were acquired with the same parameters as the images of interest. Nuclear/cytoplasmic ratios were analyzed using Zeiss LSM 510 software. For each cell analyzed, the nuclear and cytosolic densities of NFATc3 and NFATc4 staining were determined as the sum of the pixels within nuclear or cytosolic regions normalized to the corresponding nuclear or cytosolic areas. Measurements were repeated in 5 Z-stacks showing the maximum nuclear area and mean densities were calculated in each cell. ToPro3 staining was used to determine nucleus contours. Results are presented as the ratio of the mean nuclear staining density to the mean cytosolic staining density.
Data Acquisition and Analysis

Clampfit 6.0 (Axon) and GraphPad Prism 3.0 were used for data analysis. Nonlinear least-square curve-fitting algorithms were performed for curve fitting. Real-time PCR results were analyzed with MXPro software from Stratagene. Western blot results were analyzed with Quantity One software (Biorad). Statistical comparisons were performed with paired or unpaired student $t$-tests if only 2 group means were compared. When multiple groups were studied simultaneously, group comparisons were performed with ANOVA. If significant differences were indicated by analysis of variance, $t$-tests with Bonferroni’s correction or Dunnett’s tests were used to evaluate differences between individual mean values. A two-tailed $P<0.05$ was taken to indicate statistical significance. Data are expressed as mean±SEM.
References


Online Table I

Properties of 1-Hz and 3-Hz paced cells

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<th>Rs</th>
<th>Capacitance</th>
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<td>n pF</td>
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<td>5.1±0.3</td>
<td>34</td>
</tr>
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</table>

RP=Resting membrane potential, Rs=compensated series resistance value, n=number of cells.  P=NS, 1-Hz versus 3-Hz groups, for all variables.
### On-line Table II

**Primer and probe information**

<table>
<thead>
<tr>
<th>Primers and Probes</th>
<th>Sequences</th>
<th>GeneBank#</th>
</tr>
</thead>
</table>
| **Kv4.3**          | F: TCCCTGTTATCTGTACGAACCT  
                     | R: TTCTGCTCAAACATCTGCTCATCT  
                     | Probe: CCACCATCAAGAACCA | XM_845974 |
| **Kv1.4**          | F: CCTCCCCTCTTAATTGCTCAAGAAA  
                     | R: TCGGAGCTCTACTTCTTC  
                     | Probe: TCGGAGCTCTACTTCTTC | XM_542545 |
| **KChIP2**         | F: CCTCAACAAGGATGGCTGCAT  
                     | R: AGGTATACTTGCCCCATCATGTCATAGA  
                     | Probe: CCAAGGAGGAAATG | XM_845759 |
**Online Figure Legends**

**Online Figure I.**  I\textsubscript{to} frequency-dependence, determined from the ratio of current during the 15\textsuperscript{th} pulse to current during the first pulse of a train of 100-ms depolarizations from -80 mV to +50 mV at the frequencies indicated. Data are mean±S.E.M (n=5 cells in 1-Hz, and n=6 cells in 3-Hz conditions).

**Online Figure II.**  A, Examples of I\textsubscript{to} recordings obtained with 100-ms depolarizations from -50 to +40 mV at 0.1 Hz, from CTL and 24-hour VTP dog cells. B, Mean±S.E.M. I\textsubscript{to} densities for epicardial myocytes freshly isolated from control (CTL, n=13 cells) and 24-hour VTP (n=19 cells) dogs. *P<0.05, **P<0.01, CTL versus 24-hour VTP.

**Online Figure III.**  A, Examples of I\textsubscript{to} recordings obtained with 100-ms depolarizations from -50 to +40 mV at 0.1 Hz, from 1-Hz and 3-Hz paced cells incubated under control (CTL) or blebbistatin (BBST, 5-μmol/L) conditions throughout the pacing period. Cell activation and mechanical uncoupling in the presence of BBST were verified by recording Ca\textsuperscript{2+}-transients (Indo-1 AM) and cell-shortening (video-imaging). B, Mean±S.E.M. I\textsubscript{to} densities at +40 mV of 1-Hz CTL and BBST, 3-Hz CTL and BBST groups, n=8 cells/group. *P<0.05, 1 Hz versus 3 Hz.

**Online Figure IV.**  A, Representative I\textsubscript{to} recordings obtained with 100-ms depolarizations from -50 to +40 mV at 0.1 Hz, from 1-Hz (3 ms), 1-Hz (9 ms) and 3-Hz (3 ms) paced cells. B, Mean±S.E.M. I\textsubscript{to} density-voltage relations. **P<0.01, 3-Hz (3 ms) versus 1-Hz (3 ms) & 1-Hz (9 ms).
**Online Figure V.** A, Examples of \( I_{\text{to}} \) recordings obtained with 100-ms depolarizations from -50 to +40 mV at 0.1 Hz, from 1-Hz and 3-Hz paced cells incubated in the absence (CTL) or presence (Val) of 1-\( \mu \)mol/L valsartan (Val) throughout the pacing period. B, Mean±S.E.M. \( I_{\text{to}} \) densities for 1- (n=10 cells) and 3-Hz (n=9 cells) CTL, 1- (n=8 cells) and 3-Hz (n=10 cells) Val cells, with the recording protocol used in panel A. **\( P<0.01 \), 1-Hz versus 3-Hz cells.

**Online Figure VI.** Acute effects of 0.5-\( \mu \)mol/L nimodipine on \( I_{\text{CaL}} \). A & B, \( I_{\text{CaL}} \) recorded during the last pulse at 1 or 3 Hz before (CTL, A) and 5 minutes after addition of nimodipine (0.5 \( \mu \)mol/L, B). Currents were recorded with 10 consecutive 100-ms pulses from a holding potential of -50 mV to a depolarizing potential of +10 mV, with a 1 Hz or 3 Hz pulse frequency. C, Mean±S.E.M. percentage decrease of \( I_{\text{CaL}} \) density by 0.5-\( \mu \)mol/L nimodipine (n=3 cells). D, Mean±S.E.M. \( I_{\text{CaL}} \) densities under each condition (n=3 cells). *\( P<0.05 \) by one-way ANOVA, CTL versus nimodipine.

**Online Figure VII.** A, Mean±S.E.M. \( I_{\text{to}} \) densities obtained with 100-ms depolarizations from -50 to +40 mV at 0.1 Hz, from 1-Hz and 3-Hz paced cells incubated under control (CTL) or A-7 (1- or 5-\( \mu \)mol/L) conditions throughout the pacing period. n=7-11 cells/group for 1-Hz, n=8-9 cells/group for 3-Hz. *\( P<0.05 \), 1-Hz versus 3-Hz. B, Mean±S.E.M. \( I_{\text{to}} \) densities obtained with 100-ms depolarizations from -50 to +40 mV at 0.1 Hz, from 1-Hz and 3-Hz paced cells incubated under control (CTL), W-13 (40-\( \mu \)mol/L) or its inactive congener W-12 (40-\( \mu \)mol/L) throughout the pacing period. n=6-7 cells/group for 1-Hz, n=6-7 cells/group for 3-Hz. *\( P<0.05 \), 1-Hz versus 3-Hz.
**Online Figure VIII.** Acute effects of KN93 and KN92 on $I_{Ca\text{L}}$. Currents were recorded with 10 consecutive 100-ms pulses from a holding potential of -50 mV to a depolarizing potential of +10 mV, with a 1 Hz or 3 Hz pulse frequency. A, Top: $I_{Ca\text{L}}$ recorded during the last pulse at 3 Hz before (CTL) and 5 minutes after addition of KN93 (1 µmol/L), and 5 minutes after subsequent addition of nimodipine (0.5 µmol/L) as a positive control for each cell. Bottom: Mean±S.E.M. $I_{Ca\text{L}}$ densities under each condition (n=5 cells). B, Top: $I_{Ca\text{L}}$ recorded during the last pulse at 3 Hz before (CTL) and after addition of KN92, and 5 minutes after subsequent addition of nimodipine (0.5 µmol/L) as a positive control for each cell. Bottom: Mean±S.E.M. $I_{Ca\text{L}}$ densities under each condition. (n=4 cells).

**Online Figure IX.** A, Expanded presentation of Western blot results shown in Figures 4A, C & E. Full lanes are shown for CaMKII-phosphorylated phospholamban at the left (along with corresponding GAPDH bands) and corresponding results obtained from the same gels after stripping and re-probing for total phospholamban are shown at the right. B, Expanded presentation of gels for Western blot results shown in Figure 5D for Kv4.3 and KChIP2 from KN93 and KN92 treated cells. C, Expanded presentation of gels for Western blot results shown in Figure 6B for calcineurin. For all proteins, prominent bands were seen at the expected molecular masses.

**Online Figure X.** A, Examples of sodium-calcium exchanger (NCX, ~120 kDa), ryanodine receptor (RyR2, ~520 kDa), sarcoplasmic endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA2a, ~110 kDa, abbreviated as SERCA in figure) and
GAPDH Western blots from 1-Hz and 3-Hz paced cells. B, Mean±S.E.M. NCX, RyR2 and SERCA band intensities after normalization to GAPDH-band intensities (n=4 blots each for all proteins, each blot being from 2 hearts, total of 8 hearts/group).

**Online Figure XI.** Examples of immunolocalization of NFATc3 (red), NFATc4 (green), ToPro3 (blue) and their merged co-localization in 1- and 3-Hz paced myocytes.

**Online Figure XII. A&B, Top,** examples of NFATc3 (A, ~130kDa) and NFATc4 (B, ~160 kDa) Western blots from 1-Hz and 3-Hz paced-cell total extracts and nuclear extracts. **Bottom,** mean±S.E.M. NFATc3 and NFATc4 nuclear-enriched (fraction D) and nuclear-deficient (fraction B) band-intensities normalized to total cell-lysate (fraction A) band-intensity (n=4 blots for each protein; each blot was produced from cells exposed to 1- and 3-Hz pacing from 2 hearts each to provide enough protein from the nuclear fraction, thus providing a total of 8 hearts/observation).
Online Figure III

A

BBST = blebistatin
CTL = Control

Time (ms)

pA/pF

1-Hz CTL
1-Hz BBST
3-Hz CTL
3-Hz BBST

B

1-Hz CTL  1-Hz BBST  3-Hz CTL  3-Hz BBST

pA/pF

*
Online Figure IV

A

B

- **TP (mV)**

- **pA/pF**

- **1-Hz (3 ms)**
- **1-Hz (9 ms)**
- **3-Hz (3 ms)**

- **1-Hz (3 ms, n=12)**
- **1-Hz (9 ms, n=9)**
- **3-Hz (3 ms, n=10)**

- **ms**

- **-50** - **75**
Online Figure V

A

B

I_o at 40 mV (pA/pF)

Time (ms)

1-Hz CTL

1-Hz Val

3-Hz CTL

3-Hz Val

I_o density at +40 mV (pA/pF)

1-Hz CTL (n=10)

1-Hz Val (n=8)

3-Hz CTL (n=9)

3-Hz Val (n=10)
Online Figure VI

A

CTL

Time (ms)

N=3 cells/group

B

Nimo

Time (ms)

ICaL density at +10 mV (pA/pF)

C

D

ICaL % decrease by nimodipine

1-Hz CTL  3-Hz CTL  1-Hz Nimo  3-Hz Nimo

P=NS

1-Hz CTL  3-Hz CTL  1-Hz Nimo  3-Hz Nimo

P=NS

*
Online Figure VII

A  

![Bar chart showing pA/pF values for different conditions.](chart1)

- 1 Hz (N=7-11 cells/group)
- 3 Hz (N=8-9 cells/group)

<table>
<thead>
<tr>
<th>Condition</th>
<th>1 Hz</th>
<th>3 Hz</th>
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<tbody>
<tr>
<td>CTL</td>
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</tr>
<tr>
<td>A-7 1 μM</td>
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<td>15</td>
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<tr>
<td>A-7 5 μM</td>
<td>14</td>
<td>17</td>
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</tbody>
</table>

* P<0.05

B  

![Bar chart showing pA/pF values for different conditions.](chart2)

- 1 Hz (N=6-7 cells/group)
- 3 Hz (N=6-7 cells/group)

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<th>Condition</th>
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<tbody>
<tr>
<td>CTL</td>
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<tr>
<td>W-13 40 μM</td>
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<td>15</td>
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<tr>
<td>W-12 40 μM</td>
<td>14</td>
<td>17</td>
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</tbody>
</table>

* P<0.05
Online Figure VIII

A

B

CTL

KN93

Nimodipine

1 pA/pF

25 ms

1 Hz

3 Hz

Nimo

CTL

KN92

Nimodipine

1 pA/pF

25 ms

1st pulse

10th pulse

1st pulse

10th pulse

I_{CaL} density (pA/pF)
Online Figure IX

A. 

<table>
<thead>
<tr>
<th></th>
<th>CTL 1-Hz</th>
<th>CTL 3-Hz</th>
<th>KN93 1-Hz</th>
<th>KN93 3-Hz</th>
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B. 

<table>
<thead>
<tr>
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<th>KN93 1-Hz</th>
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<td>KChIP2</td>
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C. 

<table>
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<tr>
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<th>3-Hz</th>
<th>3-Hz+93</th>
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Online Figure X

A

1-Hz 3-Hz

NCX

RyR2

SERCA

GAPDH

B

Normalized to GAPDH (O.D.)

<table>
<thead>
<tr>
<th></th>
<th>1-Hz</th>
<th>3-Hz</th>
<th>1-Hz</th>
<th>3-Hz</th>
<th>1-Hz</th>
<th>3-Hz</th>
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<td>NCX</td>
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<td>RyR2</td>
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<td>SERCA</td>
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</tbody>
</table>

Normalized to GAPDH (O.D.)
Online Figure XI

1-Hz

3-Hz

NFATc4  NFATc3  ToPro3  Merged
Online Figure XII

A. NFATc3

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1 Hz</th>
<th>3 Hz</th>
</tr>
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<tbody>
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<td></td>
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<tr>
<td>B</td>
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<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
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</table>

1 Hz (N=4)

3 Hz (N=4)

P<0.05

~130 kDa

B. NFATc4

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1 Hz</th>
<th>3 Hz</th>
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<tbody>
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<td></td>
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</tr>
<tr>
<td>D</td>
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</tbody>
</table>

1 Hz (N=4)

3 Hz (N=4)

~160 kDa

Normalized to total lysate fraction A

* P<0.05