Cellular Signaling Underlying Atrial Tachycardia Remodeling of L-type Calcium Current

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Abstract—Atrial tachycardia (AT) downregulates L-type Ca\(^{2+}\) current (I\(_{\text{CaL}}\)) and causes atrial fibrillation–promoting electric remodeling. This study assessed potential underlying signal transduction. Cultured adult canine atrial cardiomyocytes were paced at 0, 1, or 3 Hz (P0, P1, P3) for up to 24 hours. Cellular tachypacing (P3) mimicked effects of in vivo AT: decreased I\(_{\text{CaL}}\), transient outward current (I\(_{\text{to}}\)), unchanged I\(_{\text{CaT}}\), I\(_{\text{Kr}}\), and I\(_{\text{Ks}}\), and reduced action potential duration (APD). I\(_{\text{CaL}}\) was unchanged in P3 at 2 and 8 hours but decreased by 55±6% at 24 hours. Tachypacing caused Ca\(^{2+}\) accumulation in P3 cells at 2 to 8 hours, but, by 24 hours, Ca\(^{2+}\) returned to baseline. Ca\(_{\text{i2.1}}\) mRNA expression was not altered at 2 hours but decreased significantly at 8 and 24 hours (32±4% and 48±4%, respectively) and protein expression was decreased (47±8%) at 24 hours only. Suppressing Ca\(^{2+}\)i increases during tachypacing with the I\(_{\text{CaL}}\) blocker nimodipine or the Ca\(^{2+}\) chelator BAPTA-AM prevented I\(_{\text{CaL}}\) downregulation. Calcineurin activity increased in P3 at 2 and 8 hours, respectively, returning to baseline at 24 hours. Nuclear factor of activated T cells (NFAT) nuclear translocation was enhanced in P3 cells. Ca\(^{2+}\)-dependent signaling was probed with inhibitors of Ca\(^{2+}\)/calmodulin (W-7), calcineurin (FK-506), and NFAT (INCA6): each prevented I\(_{\text{CaL}}\) downregulation. Significant APD reductions (~30%) at 24 hours in P3 cells were prevented by nimodipine, BAPTA-AM, W-7, or FK-506. Thus, rapid atrial cardiomyocyte activation causes Ca\(^{2+}\) loading, which activates the Ca\(^{2+}\)-dependent calmodulin–calcineurin–NFAT system to cause transcriptional downregulation of I\(_{\text{CaL}}\), restoring Ca\(^{2+}\)i to normal at the cost of APD reduction. These studies elucidate for the first time the molecular feedback mechanisms underlying arrhythmogenic AT remodeling. (Circ Res. 2008;103:845-854.)

Key Words: atrial fibrillation ■ electrophysiological remodeling ■ arrhythmia mechanisms ■ antiarrhythmic therapy

Atrial fibrillation (AF) is the most common clinical tachyarrhythmia, with an incidence that increases with age and a significant association with cardiovascular morbidity and mortality.1 AF causes electrophysiological changes, primarily resulting from the rapid atrial activation rates, that promote arrhythmia perpetuation.2-3 Atrial action potential (AP) duration (APD) shortening is a major contributor to refractoriness abbreviation, which is, in turn, a primary factor in AF promotion.4-5 Loss of APD and impaired AP rate adaptation are largely attributable to the associated I\(_{\text{CaL}}\) reduction.5,6

Atrial remodeling is believed to have important therapeutic implications, and there is interest in developing antiremodeling therapies,7 but this approach is limited by an insufficient understanding of underlying mechanisms to allow for the definition of molecular targets. There is indirect evidence for a role of Ca\(^{2+}\) overload in the remodeling caused by atrial tachycardia (AT).8,9 and transcriptional downregulation of the Ca\(_{\text{i2.1}}\) I\(_{\text{CaL}}\) α-subunit contributes to I\(_{\text{CaL}}\) downregulation.5,10 However, the signaling mechanisms coupling Ca\(^{2+}\) loading to I\(_{\text{CaL}}\) downregulation are poorly understood. In this study, we used an in vitro model of paced canine atrial cardiomyocytes to assess (1) whether it mimics in vivo AT-induced cellular electrophysiological remodeling; and (2) if so, what intracellular signaling mechanisms are involved.

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

Cardiomyocyte Isolation
Single canine left atrial cells were isolated with previously described methods.11 Cardiomyocytes were kept in medium 199 and concentrated by centrifugation at 500 rpm (1 minute), and cell pellets were removed for culture.

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Cell Culture
Cells were plated at low density (~10^4 cells/cm^2) onto laminin-coated (20 μg/mL) glass coverslips and maintained at 37°C. After 4 hours, dead and unattached myocytes were removed and fresh medium was added. Pacing was accomplished with square wave, 5-ms pulses. Parallel 24-hour culture studies were performed with cells subjected to 1-Hz (P1) and 3-Hz (P3) pacing and no pacing (P0). For time course studies, results with 1- and 3-Hz pacing were compared to each other at each time point and to prepacing baseline. After cell culture with or without pacing, cells were transfected to superfusion baths for ionic current or AP recording or harvested and frozen for subsequent biochemical study. In some experiments, 1 μmol/L nifedipine, 10 μmol/L BAPTA-AM, 1 μmol/L W-7, 5 μmol/L FK-506, or 10 μmol/L INCA-6 was added to the culture medium and thoroughly washed out before recording currents or [Ca^2+]i. Cell viability was measured by Trypan blue dye exclusion.

Ionic Current and AP Recording
All in vitro recordings were obtained at 37°C. The whole-cell perforated-patch technique was used to record APs and tight seal patch clamp to record ionic currents. Junction potentials averaged 15.9 mV and were corrected for APs only. For solution contents for recording of specific ionic currents and APs, see the expanded Materials and Methods section in the online data supplement.

Calcium Transients
Atrial cardiomyocytes were incubated with indo-1 AM (5 μmol/L) in 100 μmol/L pluronic F-127 and 0.5% DMSO for 3 to 5 minutes and then superfused with Tyrode’s solution. Ultraviolet light passing through a 340-nm interference filter was reflected into a ×40 oil immersion fluor objective for excitation of intracellular indo-1. Exposure of the cell to UV light was controlled with an electronic immersion fluor objective for excitation of intracellular indo-1. Nuclear and cytosolic NFATc3 and NFATc4 localization was quantified from the total number of pixels within the point-spread functions acquired with the same parameters as the images of interest. Nuclear and cytosolic NFATc3 and NFATc4 staining was quantified from the total number of pixels within the corresponding region normalized to area. Measurements were obtained in 5 Z-stacks covering the maximum nuclear area to calculate mean densities for each cell.

Quantitative Real-Time PCR
Total RNA was isolated from 100- to 300-mg atrial cardiomyocyte samples with TRIzol, followed by chloroform extraction and isopropanol precipitation, DNase treatment, and quality control with polyacrylamide gel electrophoresis. First-strand cDNA was synthesized from 2 μg of total RNA with High Capacity cDNA Archive Kits. Real-time quantitative PCR (QPCR) was performed with 6-carboxy-fluorescein–labeled fluorescent Ca2+, TaqMan primers and probes and TaqMan universal master mix. Fluorescence signals were detected in duplicate, normalized to 18S ribosomal RNA, and quantified with MxPro QPCR software.

Western Blot Analysis
Paced atrial cardiomyocyte samples were homogenized in radioimmunoprecipitation assay buffer as previously described. The homogenate was centrifuged (15 000 rpm, 20 minutes, 4°C). The supernatant was used for protein concentration measurement by Bradford assay with BSA as a standard. For α/γ subunit assessment, 40-μg protein samples were separated by 8% Na-dodecylsulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and incubated with rabbit anti–α-cardiac Ca1.2, 1:100 and mouse antityrosylcaldelyde-3-phosphate dehydrogenase (GAPDH) (1:20 000), followed by goat anti-mouse (1:2000) or goat anti-rabbit (1:5000) horseradish peroxidase–conjugated secondary antibodies. Signals were visualized with Western Lightning Chemiluminescence Reagent Plus and quantified by videodensitometry.

Data Analysis
Clampfit 9.2, GraphPad Prism 4.0, IgorPro5.04B, MxPro-Mx 3000P, SPSS, and Origin 5.0 were used for data analysis. All data are expressed as means±SEM. Multiple group statistical comparisons were obtained by 2-way ANOVA, and individual group mean differences were evaluated by Student’s t tests with Bonferroni correction. A 2-tailed P<0.05 was considered statistically significant.

Results

AP Changes
Cell capacitance averaged 142±7, 144±8, and 148±8 pF (n=35 per group; P=NS) in P0, P1, and P3 cells, respectively. Cell viability averaged ~50% in P0 and P1 cells and was reduced to ~30% in P3 cells (supplemental Table I). Cell dimensions (supplemental Table I) and morphologies (online data supplement, Figure 1) did not change with tachypacing. After 24 hours of pacing at 0, 1, or 3 Hz, APs were recorded at frequencies between 0.1 and 2 Hz, allowing 1 minute at each frequency to reach steady state. Resting membrane potential and AP amplitude were not altered by tachypacing. Resting membrane potential averaged −72.5±2.1 mV (n=14 cells) in P0 cells compared with −74.1±1.5 mV (n=16) and −74.1±0.9 mV (n=19) in P1 and P3 cells, respectively (P=NS). AP amplitude at 1 Hz averaged 114±2 mV (n=14 cells) in P0 cells, 112±2 mV (n=16) in P1 cells, and 108±2 mV (n=19) in P3 cells (P=NS). Figure 1A shows representative APs recorded at 1 Hz from P0, P1, and P3 cardiomyocytes. APD90 (APD to 90% repolarization) was significantly reduced and APD rate adaptation blunted in P3 cells (Figure 1B). APDs were not significantly different in P0 versus P1 cells.

Ionic Current Changes
Ca2+ Currents
Figure 2A shows I_{CaL} recordings on 200-ms depolarizing steps from −50 to +10 mV. I_{CaL} density was significantly...
increased by 24-hour tachypacing at test potentials between -10 mV and +40 mV (Figure 2B). For example, $I_{Ca}$ at +10 mV averaged $-5.0 \pm 0.5$ pA/pF (n = 13), $-4.8 \pm 0.6$ pA/pF (n = 16), and $-2.0 \pm 0.1$ pA/pF (n = 13) in P0, P1, and P3 cells, respectively. $I_{Ca}$ inactivation voltage dependence was determined with 1000-ms prepulses to voltages between -90 and +50 mV, followed by 300-ms test pulses to +10 mV. Activation voltage dependence was obtained from data obtained as illustrated in Figure 2B, based on the relation $I_s = I_{max}(V-V_s)(G_s/G_{max})$, where $I_s$ and $G_s$ are current and conductance at voltage $V_s$; $I_{max}$ and $G_{max}$ are maximum current and conductance, and $V_s$ is the reversal potential. $V_s$ was determined from the horizontal axis intercept of the ascending limb of the $I_{Ca}$–voltage relation. The voltage dependencies of $I_{Ca}$ activation and inactivation were unaffected (Boltzmann fits) in tachypaced cells (Figure 2C). Voltage ($V_{1/2}$) for half-maximum activation averaged $-3.1 \pm 0.7$ (n = 13), $-3.5 \pm 0.9$ (n = 16), and $-3.6 \pm 0.8$ mV (n = 13) in P0, P1, and P3 cells, respectively (P=NS). The inactivation $V_{1/2}$ averaged $-19.3 \pm 0.8$ (n = 11), $-18.8 \pm 1.4$ (n = 12), and $-17.4 \pm 0.5$ mV (n = 12) in P0, P1, and P3 cells, respectively (P=NS). Biexponential $I_{Ca}$ inactivation time constants were unchanged by pacing (Figure 2D). Time-dependent recovery was well fitted by monoeponential functions, with time constants averaging 46.9±2.4 ms (n = 12 cells), 41.9±2.2 ms (n = 13), and 42.7±4.1 ms (n = 10) in P0, P1, P3 cells (P=NS; Figure 2E).

Records used to obtain low-voltage-activated T-type calcium current ($I_{Ca}$) are shown in supplemental Figure IIA. As previously described, $I_{Ca}$ was analyzed by subtracting currents recorded with a holding potential of -90 mV from currents in the same cell at a holding potential of -50 mV. Mean $I_{Ca}$ density–voltage relations are shown in supplemental Figure IIB. $I_{Ca}$ density was unaffected by changing pacing frequency during the 24-hour culture period. For example $I_{Ca}$ at -20 mV averaged $-1.30 \pm 0.1$ pA/pF (n = 15), $-1.25 \pm 0.1$ pA/pF (n = 20), and $-1.28 \pm 0.1$ pA/pF (n = 19) in P0, P1, and P3 cells, respectively (P=NS). Current–voltage relations showed no differences among groups, suggesting no differences in voltage dependence.

**Transient Outward Current**

$I_o$ recordings from P0, P1, and P3 cells are shown in supplemental Figure IIIA. $I_o$ was significantly reduced by tachypacing (supplemental Figure IIB). For example, $I_o$ at +40 mV averaged 7.6±0.5 pA/pF, 7.6±0.6 pA/pF (n = 18), and 3.5±0.4 pA/pF (n = 19) in P0, P1, and P3 cells, respectively. The overall form of the current–voltage relation did not change. The approach used in Figure 2C was applied to determine $I_o$ activation voltage dependence, with the $I_o$ reversal potential based on tail currents following 2.2-ms activating pulses to +50 mV averaging -71±2 mV, -73±4 mV, -70±2 mV (n=5 cells per group) in P0, P1, P3 cells (P=NS). Activation $V_{1/2}$ based on data fits in each experiment (supplemental Figure IIIIC) averaged 9.5±1.6 (n=17), 9.4±1.1 (n=15) and 10.7±1.9 mV (n=13) in P0, P1, and P3 cells (P=NS). Inactivation voltage dependence was studied with 1000-ms prepulses from -70 mV, followed by 200-ms test pulses to +60 mV. Boltzmann fit inactivation $V_{1/2}$ averaged -24.9±1.2 (n=22 cells), -26.7±0.9 (n=21), and -26.7±1.4 mV (n=19) in P0, P1, and P3 cells (P=NS) (supplemental Figure IIIIC). The time constants of $I_o$ inactivation curves, which were well fitted by monoeponential relations, showed no differences (supplemental Figure IIIID).

**Inward Rectifier and Delayed Rectifier K+ Currents**

Supplemental Figure IIC shows 1 mmol/L Ba2+–sensitive $I_{Kr}$ density–voltage relations, which were comparable among P0, P1, and P3 cells. Supplemental Figure IID and IIE shows current–voltage relations for E-4031–resistant $I_{Kr}$ and HMR1566-resistant $I_{Kr}$ tail currents in P0, P1, and P3 cells. There were no significant differences in $I_{Kr}$ or $I_{Kr}$ of P0, P1, and P3 cells.

**Intracellular Calcium Responses**

The results presented in Figures 1 and 2 and supplemental Figures II and III suggest that the isolated cell model recapitulates the principal cellular electrophysiological features of atrial tachycardia remodeling: reduced APD and APD rate adaptation, decreased $I_{Ca}$ and $I_{Kr}$, and unchanged $I_{Ca}$, $I_{Kr}$, and $I_{Kr}$.$^4$ A commonly accepted explanation of the mechanism of atrial tachycardia remodeling invokes a negative-feedback response to protect against Ca2+ overload via the downregulation of $I_{Ca}$. However, no direct evidence...
to support this notion has been presented. Our system allows for the measurement of \([\text{Ca}^{2+}]_i\), and \(I_{\text{CaL}}\) as a function of in vitro pacing duration, permitting an evaluation of the relative changes in each component. We therefore recorded \([\text{Ca}^{2+}]_i\), and \(I_{\text{CaL}}\) in parallel sets of cells from the same batches that were either not tachypaced (P1) or were paced at 3 Hz (P3) for 2, 8, or 24 hours. Figure 3A shows examples of \([\text{Ca}^{2+}]_i\), transients measured at 1 Hz from cells that had been subjected to 3-Hz pacing for 8 and 24 hours. Both diastolic and systolic \([\text{Ca}^{2+}]_i\), were greatly increased at 8 hours and returned toward baseline values after 24 hours. Mean peak systolic and diastolic \([\text{Ca}^{2+}]_i\), are shown in Figure 3B and 3C and indicate statistically significant increases with 3-Hz pacing versus prepacing baseline and P1 cells at 2 and 8 hours, with a return to values not significantly different from baseline at 24 hours. The \([\text{Ca}^{2+}]_i\), transient decay time constant decreased at 2 and
of Western blots in supplemental Figure VI), by 47±8%, in P3 cells. The changes in Ca,1,2 mRNA and protein expression were specifically related to tachypacing because neither was altered significantly in P1 cells (Figure 4, left).

**Analysis of Ca^{2+}-Dependent Pathways**

The data described above point to a Ca^{2+}-dependent pathway that is triggered by increased [Ca^{2+}i], leading to reductions in Ca,1,2 mRNA within 8 hours, which results in decreased Ca,1,2 protein expression and diminished \( I_{\text{Ca}} \) within 24 hours. If this presumed system is correct, prevention of intracellular Ca^{2+}i loading in response to 3-Hz pacing should prevent \( I_{\text{Ca}} \) downregulation. We tested this idea in 2 ways. First, we exposed P0, P1, and P3 cells throughout 24-hour pacing to 1 \( \mu \text{mol/L} \) nimodipine to suppress Ca^{2+}i entry via \( I_{\text{Ca}} \). Consistent with the hypothesized role of Ca^{2+}i entry in \( I_{\text{Ca}} \) downregulation, nimodipine prevented \( I_{\text{Ca}} \) decreases in P3 cells (Figure 5A). In additional studies, we added 10 \( \mu \text{mol/L} \) BAPTA-AM to the culture medium for the 24-hour-pacing period to buffer \([\text{Ca}^{2+}i] \). Once again, \( I_{\text{Ca}} \) downregulation was prevented in P3 cells (Figure 5B). Nimodipine and BAPTA-AM also controlled intracellular Ca^{2+}i loading, as indicated by Ca^{2+}i imaging (supplemental Figure VII).

We then turned our attention to potential Ca^{2+}-dependent signaling pathways. Calmodulin is a key Ca^{2+}-binding protein that senses intracellular Ca^{2+}i concentration and modulates a wide range of Ca^{2+}-dependent enzyme systems.16 We used the calmodulin inhibitor W-7 to address the potential importance of calmodulin in coupling \([\text{Ca}^{2+}i] \), increases to downstream effectors. Atrial cardiomyocytes were subjected to P0, P1, or P3 conditions for 24 hours in the presence of 1 \( \mu \text{mol/L} \) W-7. Calmodulin inhibition completely suppressed \( I_{\text{Ca}} \) downregulation in P3 cells (Figure 5C). Furthermore, calmodulin inhibition prevented downregulation of Cav1.2 transcript expression by tachypacing (supplemental Figure VIII A).

Calcinurin is a Ca^{2+}-dependent protein phosphatase that plays a key role in a variety of cardiac-remodeling processes, including the regulation of ion channel expression.18 We studied potential calcineurin involvement by adding the calcineurin blocker FK-506 (5 \( \mu \text{mol/L} \)) to the medium of P0, P1, and P3 cells during 24-hour pacing. FK-506 prevented \( I_{\text{Ca}} \) downregulation (Figure 5D), as well as tachypacing-induced Cav1.2 transcript downregulation (supplemental Figure VIII B). We then measured calcineurin-related phosphatase activity over time in cells exposed to 1- or 3-Hz pacing. No significant changes in calcineurin activity were observed in P1 cells (Figure 4C). In contrast, calcineurin activity was increased significantly at 2 and 8 hours of tachypacing in P3 cells. This time course indicates that calcineurin activity is rapidly increased by intracellular Ca^{2+}i loading and that calcineurin activity increases precede decreases in Ca,1,2 mRNA, consistent with a calcineurin-dependent signal that causes downregulation in mRNA expression.

Calcinurin dephosphorylates cytoplasmic NFAT transcription factors, promoting their translocation into the nucleus, where they participate in transcriptional regulation.19 We used deconvolved confocal microscopic immunofluo-
cent images to quantify cellular localization of the NFAT isoforms NFATc3 and NFATc4. Figure 6A shows examples of NFATc3, NFATc4, and merged images (with ToPro3 to define the nucleus) in P1 and P3 cells. Figure 6B shows quantification of the nuclear/cytoplasmic staining ratios. Whereas P1 and P0 cells showed similar nuclear/cytoplasmic staining, P3 cells showed stronger nuclear staining localization, consistent with nuclear translocation. If NFAT-dependent tran-
scription changes are important in $I_{\text{CaL}}$ downregulation, NFAT inhibition should suppress tachypacing-induced $I_{\text{CaL}}$ downregulation. We, therefore, added the cell-permeable NFAT inhibitor INCA-6 (10 μmol/L) to the culture medium during 24-hour pacing. Consistent with an important role for calcineurin-dependent NFAT changes, INCA-6 completely prevented $I_{\text{CaL}}$ downregulation (Figure 5E). For INCA-6, as for all of the interventions we studied, neither $I_{\text{CaL}}$ inactivation kinetics (supplemental Figure IV) nor voltage dependence (supplemental Table II) was altered with cell pacing. Consistent with nuclear NFAT translocation playing a key downstream role in Cav1.2/$I_{\text{CaL}}$ downregulation, interventions acting higher up in the signaling cascade (BAPTA-AM, W-7, and FK-506) all suppressed NFAT translocation (Figure 6C through 6E). Interestingly, despite the fact that W-7 and FK-506 prevented transcriptional downregulation of $I_{\text{CaL}}$, they did not adversely affect cell Ca$^{2+}$ indices; on the contrary, they prevented Ca$^{2+}$ transient increases with 8-hour P3 pacing (supplemental Figure IX).

**Prevention of APD Changes**

One potential interest of delineating the signal transduction processes involved in rate-dependent remodeling would be to design new strategies to prevent arrhythmogenic APD shortening. As a proof of principle, we studied the effects on cultured-myocyte APD of inhibiting various components of the signaling system. Figure 7A shows APs recorded at 1 Hz from P1 and P3 cells exposed to nimodipine, BAPTA-AM, W-7, or FK-506 throughout the 24-hour pacing period. Figure 7B shows corresponding mean ± SEM data for P0, P1, and P3 cells. The results indicate that prevention of Ca$^{2+}$ loading, suppression of calmodulin signaling, or inhibition of calcineurin prevents tachycardia-induced APD abbreviation. There might be concern that prevention of $I_{\text{CaL}}$ downregulation could have negative effects on cell viability by enhancing Ca$^{2+}$ overload. On the contrary, interventions that prevented $I_{\text{CaL}}$ downregulation were found also to prevent the deterioration in cell viability (supplemental Table I) and Ca$^{2+}$ overload (supplemental Figure VII and IX) caused by tachypacing.

**Discussion**

In the present study, we explored the cell-signaling that causes APD abbreviation and $I_{\text{CaL}}$ downregulation in an in vitro model of atrial tachycardia remodeling. The results suggest that $I_{\text{CaL}}$ downregulation results from a molecular feedback system that detects intracellular Ca$^{2+}$ loading via the Ca$^{2+}$/calmodulin system, which activates calcineurin, which, in turn, stimulates nuclear translocation of NFAT, resulting in transcriptional downregulation of Ca,1,2 that decreases Ca$^{2+}$ load by reducing $I_{\text{CaL}}$, with APD reduction as a consequence.

**Comparison With Previous Studies of Atrial Tachycardia Remodeling**

Decreased $I_{\text{CaL}}$ is a consistent feature of atrial tachycardia remodeling and is believed to contribute importantly to the APD abbreviation that shortens refractoriness and promotes AF.4,6,20 $I_{\text{CaL}}$ reduction is associated with decreased Ca,1,2 mRNA expression,5,10,15,21 and many investigators have also noted decreases in Ca,1,2 protein expression.10,21,22 A role for Ca$^{2+}$ loading in atrial tachycardia remodeling was first suspected based on the protective effects of $I_{\text{CaL}}$ blockers against short-term remodeling.8,23 Subsequent work showed that atrial cardiomyocyte Ca$^{2+}$ loading begins within several minutes of the onset of cellular tachypacing.24 Cell ultrastructure changes point to transient Ca$^{2+}$ overload in atria of goats.
with AF. Short-term tachypacing of rat atria augments nuclear NFAT expression and causes changes in BNP and c-fos expression, effects that are prevented by cyclosporin-A. Bukowska et al showed calcineurin expression and activity upregulation in atrial tissues from AF patients, in association with nuclear translocation of NFAT and increased hypertrophic gene expression. Calcineurin-related changes were reproduced by ex vivo pacing (2 to 4 Hz) of human atrial tissue slices. Lin et al presented additional evidence for in vivo activation of the calcineurin and NFAT pathways in an atrial tachypacing porcine AF model.

These previous studies suggest that atrial tachycardia causes Ca\textsuperscript{2+} loading, induces transcriptional downregulation of Ca\textsubscript{v1.2}, and can activate calcineurin-dependent mechanisms. Our study is the first to demonstrate directly causative links between atrial cardiomyocyte tachycardia, cellular Ca\textsuperscript{2+} loading, I\textsubscript{CaL} downregulation, calcineurin activation, NFAT translocation, and associated APD alterations. A schematic representing our findings is presented in Figure 8. The solid vertical arrows indicate directly measured changes in [Ca\textsuperscript{2+}]\textsubscript{i}, Cav1.2 mRNA and protein, I\textsubscript{CaL}, calcineurin activity, nuclear NFAT localization, and APD. Experiments with selective inhibitors (indicated in boxes) confirm important mediating roles for free [Ca\textsuperscript{2+}]\textsubscript{i}, Ca\textsubscript{v1.2} mRNA and protein, I\textsubscript{CaL}, calcineurin activity, nuclear NFAT localization, and APD. Experiments with selective inhibitors (indicated in boxes) confirm important mediating roles for free [Ca\textsuperscript{2+}]\textsubscript{i}, calmodulin, calcineurin, and NFAT. Our time course studies suggest the presence of an intracellular molecular feedback loop initiated by [Ca\textsuperscript{2+}]\textsubscript{i} accumulation, which leads to downregulation of Ca\textsubscript{v1.2} mRNA, followed by decreased Ca\textsubscript{v1.2} protein expression, which downregulates I\textsubscript{CaL} and mitigates [Ca\textsuperscript{2+}]\textsubscript{i} loading.

### Ca\textsuperscript{2+}/Calmodulin and Calcineurin-Mediated Ion Channel Regulation

The Ca\textsuperscript{2+}/calmodulin system is an important cardiomyocyte Ca\textsuperscript{2+} sensor that responds to intracellular Ca\textsuperscript{2+} changes on both a beat-to-beat and tonic basis. Ca\textsuperscript{2+}/calmodulin binding activates a variety of downstream mediators, including calcineurin and Ca\textsuperscript{2+}/calmodulin kinase II. Neonatal rat cardiomyocyte tachystimulation activates calcineurin/NFAT signaling. Calcineurin is a Ca\textsuperscript{2+}-activated serine/threonine phosphatase composed of a catalytic A subunit (56 to 63 kDa) that dephosphorylates a number of cytoplasmic proteins, including the regulatory domains of NFATc3 and NFATc4, which translocate into the nucleus and regulate gene transcription. NFAT is a particularly important mediator of responses to changes in intracellular Ca\textsuperscript{2+} patterns. Calcineurin/NFAT signaling alters transcriptional regulation of I\textsubscript{CaL}, with most studies showing I\textsubscript{CaL} downregulation, although upregulation has also been observed in neonatal rat cardiomyocytes. In calcineurin-overexpressing mice, I\textsubscript{CaL} is increased in calcineurin-overexpressing mice, but this effect appears to be related to hypertrophy rather than to direct effects on the I\textsubscript{CaL} system. We did not observe hypertrophic responses in tachystimulated atrial cardiomyocytes.

### Considerations of the Model

Our model reproduced many features of atrial tachycardia remodeling observed in atrial tissue samples obtained from AF patients or tachypaced animals such as dogs, pigs, and sheep, including reduced APD and APD rate dependence; reduced I\textsubscript{CaL} and I\textsubscript{Ks}; and unchanged I\textsubscript{CaT}, I\textsubscript{Kr}, and I\textsubscript{K1.2}. The solid vertical arrows indicate observed changes; inhibitors that we found to prevent I\textsubscript{CaL} and/or APD changes are shown in boxes.

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**Figure 8.** Schematic representation of our findings. Solid vertical arrows indicate observed changes; inhibitors that we found to prevent I\textsubscript{CaL} and/or APD changes are shown in boxes.
Previous in vitro models of atrial tachycardia remodeling have included rapidly paced HL-1 cells derived from a mouse atrial tumor cell line,\(^{3,8}\) and paced human atrial tissue slices.\(^{25}\) The HL-1 cell model displays important features of atrial remodeling but is limited by a low expression rate of \(I_{\text{CaL}}\) (<20% of cells display prominent \(I_{\text{CaL}}\)) and \(K^+\) channel remodeling (unchanged \(I_{\text{Ks}}\) increased \(I_{\text{Kr}}\))\(^{3,18}\) that differs from the established pattern of in vivo atrial tachycardia remodeling (reduced \(I_{\text{CaL}}\), uncharged \(I_{\text{Kr}}\)).\(^{4,20,37}\) The high tachypaced atrial slice model reproduces some biochemical features seen in AF patients, but its electrophysiological properties have not been established, and atrial tissue superfusion is known to cause important functional alterations and viability problems.\(^{39}\) One potentially important change that we did not observe in tachypaced cardiomyocytes was inward rectifier \(K^+\) current enhancement.\(^{37}\) A possible explanation for this is that inward rectifier \(K^+\) current enhancement develops more slowly than the other changes, not having been noted with <7-day atrial tachyarrhythmia.\(^{40}\)

Tachypaced cardiomyocytes in our model did not show cellular hypertrophy, consistent with previous studies of atrial cells from dogs subjected to up to 42-day tachypacing.\(^{4}\) Atrial cells from patients with persistent AF may be hypertrophied,\(^{6}\) possibly because of underlying cardiac disease or the effects of greater AF chronicity, a difference that must be considered in interpreting our findings.

**Potential Significance of Our Observations**

To our knowledge, our study is the first to explore in detail the molecular signaling underlying atrial tachycardia cellular electrophysiological remodeling. Atrial tachycardia remodeling has significant clinical consequences.\(^{2,3}\) Suppression of atrial remodeling is a promising target for AF therapy development,\(^{7,41}\) but a major limitation to therapeutic innovation in this area is a lack of clear information about underlying molecular mechanisms. The cellular signaling underlying atrial tachycardia remodeling defined in the present study is, therefore, of considerable potential significance. Furthermore, we have shown that inhibition of key steps in the signaling chain can prevent remodeling-induced AP changes that are important in AF promotion. Interestingly, interventions that suppressed \(I_{\text{CaL}}\) downregulation improved \(Ca^{2+}\) loading and cell viability, suggesting that the signaling cascade producing \(I_{\text{CaL}}\) downregulation may not effectively prevent \(Ca^{2+}\) overload and cell damage and may actually produce counterproductive consequences. This is a novel observation with potentially important implications for the management of AF and the development of antiremodeling therapies. In addition to identifying potential targets for antiremodeling therapy, we have established a cellular model of atrial tachycardia remodeling that may be useful for further investigations of the underlying cellular and molecular biology.

**Acknowledgments**

We thank Chantal St-Cyr and Nathalie L’Heureux for technical assistance, France Thériault for secretarial support, and Balazs Ordog for help with QPCR.

**Sources of Funding**

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**Disclosures**

None.

**References**


Cellular Signaling Underlying Atrial Tachycardia Remodeling of L-type Calcium Current
Xiao Yan Qi, Yung-Hsin Yeh, Ling Xiao, Brett Burstein, Ange Maguy, Denis Chartier, Louis R. Villeneuve, Bianca J.J.M. Brundel, Dobromir Dobrev and Stanley Nattel

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Online Materials and Methods

Cardiomyocyte Isolation

Single canine left-atrial cells were isolated with previously-described methods. All animal care procedures followed NIH guidelines and were approved by the Animal Research Ethics Committee of the Montreal Heart Institute. Adult mongrel dogs (20-30 kg) were anesthetized with morphine (2 mg/kg s.c.) and α-chloralose (120 mg/kg i.v.) and mechanically ventilated. The heart was removed after intra-atrial injection of heparin (10,000 U), immersed in 2 mmol/L Ca²⁺-containing Tyrode’s solution, the left coronary artery was cannulated, and left-atrial tissue perfused with Tyrode’s solution (37°C, 100% O₂), then with Ca²⁺-free Tyrode’s solution (~5 minutes), followed by ~40-minute perfusion with the same solution containing collagenase (~0.4 mg/mL, CLSII, Worthington) and 0.1% bovine serum albumin (BSA, Sigma). Tissues were minced and atrial cardiomyocytes harvested. Cardiomyocytes were kept in medium-199, concentrated by centrifugation at 500 rpm (1 min), and cell-pellets removed for culture.

Cell-culture

Cells were plated at low density (~10⁴ cells/cm²) onto laminin-coated (20-μg/mL) glass coverslips (Nalge Nunc International) and maintained at 37°C in a humidified, 5% CO₂-enriched atmosphere. Medium-199 (GIBCO-BRL) was supplemented with 1% penicillin/streptomycin (Invitrogen) and 1% insulin-transferrin-selenium-X for cell-culture. After 4 hours, dead and unattached myocytes were removed and fresh medium was added.² Pacing was accomplished with square-wave, 5-ms pulses (C-pace Cell-Culture Stimulator, IonOptix). For each set of 24-hour culture experiments, parallel studies were performed with cells cultured in the presence of 1-Hz (P1) and 3-Hz (P3) pacing and no pacing (P0). For time-course studies, results with
1 and 3-Hz pacing were compared to each other at each time-point and to pre-pacing baseline. Cell contraction was verified immediately after pacing onset and then at least thrice more during 24-hour pacing (approximately 2, 8 and 24 hours after pacing-onset), both visually and by cell contraction recordings, and ensured that over 90% of viable cells followed 1:1. After cell-culture with or without pacing, cells were transferred to superfusion-baths for ionic-current or AP recording, or harvested and frozen for subsequent biochemical study. In some experiments, 1-µmol/L, nimodipine, 10-µmol/L BAPTA-AM, 1-µmol/L W-7, 5-µmol/L FK-506, or 10-µmol/L INCA-6 was added to the culture medium for the 24-hour pacing-period, and thoroughly washed out before measuring currents or [Ca^{2+}]. Cell-viability was determined by incubating cells in 0.4% Trypan-blue in Tyrode solution and counting the number of cells containing the dye vs those excluding it, with a hemocytometer.

**Ionic-current and AP Recording**

All *in-vitro* recordings were obtained at 37°C. The whole-cell perforated-patch technique was used to record APs in current-clamp mode and tight-seal patch-clamp was used to record currents in voltage-clamp mode. Borosilicate glass electrodes (Sutter Instrument) filled with pipette solution were connected to a patch-clamp amplifier (Axopatch 200A, Axon). Electrodes had tip resistances of 2 to 5 MΩ. Nystatin-free intracellular solution was placed in the tip of the pipette by capillary action (30 s), then pipettes were back-filled with nystatin-containing (600-µg/mL) pipette solution. Currents are expressed as densities (pA/pF). Junction potentials between bath and pipette solutions averaged 15.9 mV and were corrected for APs only.

Tyrode’s solution contained (in mmol/L) NaCl 136, CaCl2 1.8, KCl 5.4, MgCl2 1, NaH2PO4 0.33, dextrose 10, and HEPES 5, titrated to pH 7.4 with NaOH. The pipette solution for AP
recording contained (in mmol/L) GTP 0.1, potassium-aspartate 110, KCl 20, MgCl2 1, MgATP 5, HEPES 10, sodium-phosphocreatine 5, and EGTA 0.05 (pH 7.4, KOH). For delayed-rectifier current recording, nifedipine (10-µmol/L), 4-aminopyridine (2-mmol/L), and atropine (200-nmol/L) were added to suppress $I_{CaL}$, transient-outward current ($I_{to}$), and acetylcholine-regulated K$^+$-current ($I_{KACCh}$), respectively. E-4031 (5-µmol/L) was added for slow delayed-rectifier ($I_{Ks}$) recording. HMR1566 (500-nmol/L) was added for rapid delayed-rectifier ($I_{Kr}$) recording. For $I_{to}$ and inward-rectifier ($I_{K1}$) recording, nifedipine was replaced by CdCl2 (200-µmol/L). $I_{to}$ was studied in the presence of 10-mmol/L tetraethylammonium to inhibit the ultrarapid delayed rectifier current. $I_{K1}$ was recorded as the 1-mmol/L Ba$^{2+}$-sensitive current. The internal solution for K$^+$-current recording contained (in mmol/L) potassium-aspartate 110, KCl 20, MgCl2 1, MgATP 5, LiGTP 0.1, HEPES 10, sodium-phosphocreatine 5, and EGTA 5.0, titrated to pH 7.3 with KOH.

The extracellular solution for $I_{Ca}$ measurement contained (in mmol/L) tetraethylammonium chloride 136, CsCl 5.4, MgCl2 1, CaCl2 2, NaH2PO4 0.33, dextrose 10, and HEPES 5, titrated to pH 7.4 with CsOH. Niflumic acid (50-µmol/L) was added to inhibit Ca$^{2+}$-dependent Cl$^-$-current, and 4-aminopyridine (2-mmol/L) was added to suppress $I_{to}$. The pipette solution for $I_{Ca}$-recording contained (mmol/L) CsCl 120, tetraethylammonium chloride 20, MgCl2 1, EGTA 10, MgATP 5, HEPES 10, and Li-GTP 0.1, titrated to pH 7.4 with CsOH.

**Calcium-transients**

To record Ca$^{2+}$-transients, atrial cardiomyocytes were incubated with Indo-1 AM (5-µmol/L, Molecular Probes) in 100-µmol/L pluronic F-127 (Molecular Probes) and 0.5% DMSO (Sigma) for 3-5 min, then superfused with Tyrode’s solution for at least 20 minutes to wash out
extracellular indicator and to allow for de-esterification. Ultra-violet light from a 100-W mercury arc lamp passing through a 340-nm interference filter (±10 nm bandwidth) was reflected by a dichroic mirror into a ×40 oil-immersion fluor objective for excitation of intracellular Indo-1 (excitation beam ~15 µm diameter). Exposure of the cell to UV light (5-10 s of every 30-60 s) was controlled with an electronic shutter (Optikon) to minimize photobleaching. Emitted light (<550 nm) was reflected into a spectral separator, passed through parallel filters at 400 and 500 nm (±10 nm), detected by matched photomultiplier-tubes (Hamamatsu) and electronically filtered at 60 Hz. Chamber background fluorescence was removed by adjusting the 400-nm and 500-nm channels to zero over an empty field of view near the cell. The fluorescence-signal ratios (R400/500) were digitized (1-kHz) and converted to [Ca ] i as previously described.3 Cells were paced during Ca 2+-transient measurements with 10-ms pulses at 1.5-times threshold-voltage via two platinum electrodes separated by 2 cm.3

**Calcineurin Activity and Nuclear Factor of Activated T-cells (NFAT)-imaging**

Atrial-cardiomyocyte samples were collected pre-pacing and after 2, 8 or 24 hours of 1-Hz or 3-Hz pacing. Calcineurin phosphatase-activity was measured with a commercial assay-kit (Calbiochem). Atrial-cardiomyocyte samples were homogenized in lysis buffer. Calcineurin activity was measured in phosphatase standard buffer and determined as the dephosphorylation rate of the R-II peptide. Free phosphate released from R-II peptide was measured at 620-nm with a 96-well plate-reader (Tecan SPECTAR Rainbow).

To measure nuclear and cytoplasmic NFAT-immunofluorescence, cells were fixed with 2%-paraformaldehyde in phosphate-buffered saline (PBS), blocked and permeabilized with PBS containing 2%-normal donkey serum (NDS, Jackson), 2%-BSA and 0.1% Triton-X100. Cells
were then incubated overnight at 4°C with primary antibodies (monoclonal mouse anti-NFATc3 and polyclonal rabbit anti-NFATc4, Santa-Cruz, 1/200 in PBS with 1%-NDS, 1%-BSA and 0.05% Triton-X100). Donkey anti-mouse Alexa-Fluor (emission-peak 555-nm) and donkey anti-rabbit Alexa-Fluor (488-nm, both from Invitrogen) were corresponding secondary antibodies. Cells were then exposed to RNaseA (100-µg/mL, Roche) for 25 min at 37°C followed by 3 washes, then incubated with ToPro3 for nuclear-contour definition (1-µmol/L, Invitrogen, 647-nm emission-peak) for 45 min. Images were obtained with a Zeiss LSM-510 confocal microscopy system, and deconvolved with point-spread functions acquired with the same parameters as the images of interest. Nuclear and cytosolic NFATc3 and NFATc4-staining was quantified from the total number of pixels within the corresponding region normalized to area. Measurements were obtained in 5 Z-stacks covering the maximum nuclear area to calculate mean densities for each cell.

**Quantitative Real-time PCR**

Total RNA was isolated from 100-300 mg atrial-cardiomyocyte samples with TRIzol reagent (Invitrogen), followed by chloroform extraction and isopropanol precipitation, DNase-treatment and quality-control with polyacrylamide-gel electrophoresis. First-strand cDNA was synthesized from 2-µg total RNA with High Capacity cDNA Archive Kits (Applied Biosystems). Real-time RT-PCR was performed with 6-carboxy-fluorescein (FAM)-labeled fluorogenic Ca,1.2 TaqMan primers and probe (Assay-by-design, accession number XM_534932) and TaqMan universal master mix (Applied Biosystems) with the Stratagene Mx3000P sequence-detection system. Fluorescence-signals were detected in duplicate, normalized to 18S-ribosomal RNA (Applied Biosystems) and quantified with MxPro QPCR software (Stratagene).
**Western-blot Analysis**

Paced atrial-cardiomyocyte samples were homogenized in RadioImmuno Precipitation Assay (RIPA) buffer as previously described.\(^5\) The homogenate was centrifuged (15,000 rpm, 20 min, 4°C). The supernatant was used for protein-concentration measurement by Bradford assay (Bio-Rad) with BSA as a standard. For \(\alpha_{1c}\)-subunit assessment, 40-\(\mu\)g protein samples were separated by 8%-Na-dodecylsulfate polyacrylamide-gel electrophoresis. After transfer to nitrocellulose membranes (Bio-Rad), membranes were incubated with rabbit anti-cardiac Cav1.2, 1:100 (Alomone Labs) and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:20,000 Research Diagnostics), followed by goat anti-mouse (1:2,000, Santa-Cruz) or goat anti-rabbit (1:5,000, Santa-Cruz) HRP-conjugated secondary antibodies. Signals were visualized with Western Lightning Chemiluminescence Reagent-Plus (Perkin-Elmer Life Sciences) and quantified by videodensitometry.

**Data Analysis**

Clampfit 9.2 (Axon), GraphPad Prism 4.0, IgorPro5.04B, MxPro-Mx 3000P, SPSS and Origin 5.0 were used for data analysis. All data are expressed as mean±SEM. Multiple-group statistical comparisons were obtained by two-way ANOVA and individual group-mean differences evaluated by Student’s \(t\)-tests with Bonferroni correction. A 2-tailed \(P<0.05\) was considered statistically-significant.
References:


**Online Table I**
Canine atrial cardiomyocyte viability and cell dimensions under different experimental protocols

<table>
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<th>Live cells</th>
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<th>Length (µm)</th>
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<td>53±1%</td>
<td>18.2±0.8</td>
<td>136±5</td>
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<tr>
<td>Control P0</td>
<td>53±1%</td>
<td>18.3±0.9</td>
<td>141±4</td>
</tr>
<tr>
<td>Control P1</td>
<td>53±1%</td>
<td>18.7±0.4</td>
<td>137±1</td>
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<tr>
<td>Control P3</td>
<td>32±1%***</td>
<td>18.1±0.8</td>
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<td>Nimodipine (1 µM) P0</td>
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<td>18.1±1.0</td>
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<td>Nimodipine (1 µM) P1</td>
<td>50±1%</td>
<td>18.7±0.8</td>
<td>137±1</td>
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<td>Nimodipine (1 µM) P3</td>
<td>53±1%</td>
<td>18.1±1.0</td>
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<td>BAPTA-AM (10 µM) P0</td>
<td>50±1%</td>
<td>17.8±0.8</td>
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<td>BAPTA-AM (10 µM) P1</td>
<td>54±3%</td>
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<td>16.5±1.0</td>
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<td>W-7 (1µM) P1</td>
<td>57±1%</td>
<td>16.1±1.1</td>
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<tr>
<td>W-7 (1µM) P3</td>
<td>56±1%</td>
<td>15.6±1.1</td>
<td>142±6</td>
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<tr>
<td>FK-506 (5 µM) P0</td>
<td>53±1%</td>
<td>14.8±0.9</td>
<td>132±6</td>
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<td>FK-506 (5 µM) P1</td>
<td>52±1%</td>
<td>15.6±0.8</td>
<td>137±5</td>
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<td>FK-506 (5 µM) P3</td>
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<td>14.8±0.5</td>
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<td>INCA (10 µM) P0</td>
<td>53±1%</td>
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<td>15.0±0.4</td>
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</table>

Values are mean±SEM, ***P<0.001, P3 vs P0 and P1 Hz cells. Cell viability was determined by incubating cells in 0.4% Trypan-blue in Tyrode solution and counting the number of cells containing the dye vs those excluding it, with a hemocytometer.
Online Table II
Voltage for half-maximal inactivation (V½) and slope factor (SF)

<table>
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<th>Nimo</th>
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<th>FK-506</th>
<th>INCA-6</th>
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<tr>
<td>V½ (mV)</td>
<td>P3</td>
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<tr>
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<td>18±1.1</td>
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<tr>
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<td>17±0.3</td>
<td>17±0.7</td>
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<td>18±0.8</td>
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<tr>
<td>SF (mV)</td>
<td>P3</td>
<td>6.9±0.4</td>
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<td>8.7±0.5</td>
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<td></td>
<td>P1</td>
<td>6.7±0.3</td>
<td>7.3±0.3</td>
<td>8.2±0.6</td>
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</table>

Values are mean±SEM. There were no significant differences under any condition.
Online Figure legends

Online Figure I. Microscopic images of atrial cardiomyocytes after 24 hour culture under different experimental protocols. Horizontal scale = 10 µm.

Online Figure II. A, Representative Ca^{2+} currents elicited by double pulse protocol and method of separation of \( I_{CaL} \) versus \( I_{CaT} \). B, \( I_{Ca-T} \) densities (n=12, 12, and 15 cells in cells cultured for 24 hours under in cells cultured for 24 hours under P0, P1 and P3 conditions respectively). C, Mean±SEM \( I_{K1} \) density-voltage relations (n=9-10 cells in P0, P1, P3). D, Mean±SEM \( I_{Kr} \) tail current densities (n=10-15 cells in P0, P1, P3). E, Mean±SEM \( I_{Kr} \) tail current densities (n=10-12 cells in P0, P1, P3).

Online Figure III. A, \( I_{to} \) recordings at 0.1 Hz in cells cultured for 24 hours under P0, P1 or P3 conditions. B, Mean±SEM \( I_{to} \) density-voltage relations (n=18-20 cells/group; ***\( P<0.001 \) P3 versus P1, P0). C, Mean±SEM voltage-dependence of \( I_{to} \) inactivation and activation (n=18-29 cells/group). D, \( I_{to} \) inactivation time-constants (n=14-19 cells/group).

Online Figure IV. \( I_{CaL} \) inactivation time-constants after 24 hour culture under different experimental protocols.

Online Figure V. \( I_{CaL} \) densities of cultured cardiomyocytes pre-pacing (BL) and paced for 2, 8, or 24 hours at 1 Hz (n=10-14 cells/group).

Online Figure VI. Examples of Western blots for Cav1.2 protein (~200 kDa) and corresponding GAPDH (~36 kDa) signals.

Online Figure VII. A-C, \( Ca^{2+} \)-transient systolic, diastolic levels and decay time-constants at 1 Hz at pre-pacing baseline (BL) and in P1, P3-cardiomyocytes after pacing for 8, or 24 hours with or without nimodipine. D-F, \( Ca^{2+} \)-transient systolic, diastolic levels.
and decay time-constants at 1 Hz in BL and in P1,P3-cardiomyocytes after pacing for 8, or 24 hours with or without BAPTA-AM (*P<0.05, **P<0.01, ***P<0.001).

**Online Figure VIII.** Ca{\textsubscript{v}}.1.2 gene-expression in cells subjected to 24 hours of P1 or P3 pacing, or left unpaced (P0), in the presence of W-7 (A) or FK-506 (B).

**Online Figure IX.** A-C, Ca{\textsuperscript{2+}}-transient systolic, diastolic levels and decay time-constants at 1 Hz at pre-pacing baseline (BL) and in P1, P3-cardiomyocytes paced for 8, or 24 hours with or without W-7. D-F, Ca{\textsuperscript{2+}}-transient systolic, diastolic levels and decay tim-constants at 1 Hz in BL and in P1, P3-cardiomyocytes paced for 8, or 24 hours with or without FK-506. (*P<0.05, **P<0.01, ***P<0.001).
Online Figure I

CTL   1 μM nimodipine   1 μM W-7    10 μM INCA-6

P0

P1

P3

Online Figure I

10 μm
Online Figure II

A. Temporal profile of current density (pA/pF) as a function of TP (mV) for different membrane potentials. (a) Hyperpolarized at -50 mV (I_{CaL} only). (b) Hyperpolarized at -90 mV (I_{CaL} + I_{CaT}).

B. Voltage dependence of current density (pA/pF) for I_{CaT} as a function of TP (mV) for different membrane potentials (P0, P1, P3).

C. Voltage dependence of current density (pA/pF) for I_{K1} as a function of TP (mV).

D. Voltage dependence of current density (pA/pF) for I_{Ks} as a function of TP (mV).

E. Voltage dependence of current density (pA/pF) for I_{Kr} as a function of TP (mV).
Online Figure III
Online Figure IV

A. No drug

B. 1 µM W-7

C. 1 µM nimodipine

D. 5 µM FK-506

E. 10 µM BAPTA-AM

F. 10 µM INCA-6

Time constant (ms)

TP (mV)
Online Figure V
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<th>P1</th>
<th>P3</th>
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<td>hrs</td>
<td>0</td>
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<td>2</td>
<td>8</td>
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</table>

### Cav1.2

- 200 kDa
- 100 kDa
- 50 kDa

### GAPDH

- 36 kDa

**Online Figure VI**
Figure IX

A. 1 µM W-7

B. Diastolic [Ca2+]i nM

C. Decay time constant (ms)

D. 5 µM FK-506

E. Systolic [Ca2+]i nM

F. Decay time constant (ms)

Online Figure IX