Sex, Age, and Regional Differences in L-Type Calcium Current Are Important Determinants of Arrhythmia Phenotype in Rabbit Hearts With Drug-Induced Long QT Type 2

Carl Sims, Steven Reisenweber, Prakash C. Viswanathan, Bum-Rak Choi, William H. Walker, Guy Salama

Abstract—In congenital and acquired long QT type 2, women are more vulnerable than men to Torsade de Pointes. In prepubertal rabbits (and children), the arrhythmia phenotype is reversed; however, females still have longer action potential durations than males. Thus, sex differences in K+ channels and action potential durations alone cannot account for sex-dependent arrhythmia phenotypes. The L-type calcium current ($I_{Ca,L}$) is another determinant of action potential duration, Ca$^{2+}$ overload, early afterdepolarizations (EADs), and Torsade de Pointes. Therefore, sex, age, and regional differences in $I_{Ca,L}$ density and in EAD susceptibility were analyzed in epicardial left ventricular myocytes isolated from the apex and base of prepubertal and adult rabbit hearts. In prepubertal rabbits, peak $I_{Ca,L}$ at the base was 22% higher in males than females (6.4±0.5 versus 5.0±0.2 pA/pF; $P<0.03$) and higher than at the apex (6.4±0.5 versus 5.0±0.3 pA/pF; $P<0.02$). Sex differences were reversed in adults: $I_{Ca,L}$ at the base was 32% higher in females than males (9.5±0.7 versus 6.4±0.6 pA/pF; $P<0.002$) and 28% higher than the apex (9.5±0.7 versus 6.9±0.5 pA/pF; $P<0.01$). Apex–base differences in $I_{Ca,L}$ were not significant in adult male and prepubertal female hearts. Western blot analysis showed that Ca$_{1.2a}$ levels varied with sex, maturity, and apex–base, with differences similar to variations in $I_{Ca,L}$; optical mapping revealed that the earliest EADs fired at the base. Single myocyte experiments and Luo–Rudy simulations concur that $I_{Ca,L}$ elevation promotes EADs and is an important determinant of long QT type 2 arrhythmia phenotype, most likely by reducing repolarization reserve and by enhancing Ca$^{2+}$ overload and the propensity for $I_{Ca,L}$ reactivation. (Circ Res. 2008;102:e86–e100.)

Key Words: cardiac voltage-gated calcium current $I_{Ca,L}$ sex differences QT interval ion channel expression Torsade de Pointes

Women have longer rate-corrected QT intervals and are especially prone to QT prolongation and Torsade de Pointes (TdP) after treatment with drugs that inhibit K+ channels. A number of studies have shown an increase of TdP in women versus men following an exposure to agents known to block the K+ channel HERG and inhibit the rapid component of the delayed rectifying current, $I_{Kr}$. The increase in vulnerability to sudden death in women has been reported for cardiac and noncardiac drugs. These sex differences result most likely from the regulation of ionic channel expression by sex steroids. In the congenital form of the delayed rectifying current, $I_{Kr}$, is another determinant of action potential duration, Ca$^{2+}$ overload, early afterdepolarizations (EADs), and Torsade de Pointes. Therefore, sex, age, and regional differences in $I_{Ca,L}$ density and in EAD susceptibility were analyzed in epicardial left ventricular myocytes isolated from the apex and base of prepubertal and adult rabbit hearts. In prepubertal rabbits, peak $I_{Ca,L}$ at the base was 22% higher in males than females (6.4±0.5 versus 5.0±0.2 pA/pF; $P<0.03$) and higher than at the apex (6.4±0.5 versus 5.0±0.3 pA/pF; $P<0.02$). Sex differences were reversed in adults: $I_{Ca,L}$ at the base was 32% higher in females than males (9.5±0.7 versus 6.4±0.6 pA/pF; $P<0.002$) and 28% higher than the apex (9.5±0.7 versus 6.9±0.5 pA/pF; $P<0.01$). Apex–base differences in $I_{Ca,L}$ were not significant in adult male and prepubertal female hearts. Western blot analysis showed that Ca$_{1.2a}$ levels varied with sex, maturity, and apex–base, with differences similar to variations in $I_{Ca,L}$; optical mapping revealed that the earliest EADs fired at the base. Single myocyte experiments and Luo–Rudy simulations concur that $I_{Ca,L}$ elevation promotes EADs and is an important determinant of long QT type 2 arrhythmia phenotype, most likely by reducing repolarization reserve and by enhancing Ca$^{2+}$ overload and the propensity for $I_{Ca,L}$ reactivation. (Circ Res. 2008;102:e86–e100.)

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In rabbit models of drug-induced LQ2, adult females had significantly lower $I_{Kr}$ and, perhaps, inward rectifying K+ current, which contributed to their longer QT interval and greater arrhythmia vulnerability compared with their male counterpart. The present consensus is that normal female hearts express fewer functional K+ channels, resulting in longer action potential (AP) durations (APDs), and, when treated with agents that inhibit $I_{Kr}$, adult females have a greater vulnerability to early afterdepolarizations (EADs) and TdP. The concept of “repolarization reserve” emerged to explain the greater vulnerability of women to TdP; according to this concept, K+ channel inhibition prolongs APDs more markedly in females than males.

In prepubertal rabbit hearts with drug-induced LQT2, we showed that sex differences in arrhythmia phenotype are reversed, with males being highly vulnerable to $I_{Kr}$ blockade...
compared with females. In prepubertal (before the surge of sex hormones) rabbits (<42 days old), female hearts had longer APDs than males, yet the potnet \( I_{\text{Kr}} \) blocker E4031 failed to elicit EADs and TdP despite a marked prolongation of APDs of more than 1 second. Findings in prepubertal rabbit hearts seemed to differ from human data from children with congenital forms of LQT2.\(^{10,11} \) Analysis of human registry data revealed that adult females with congenital LQT2 had a significantly higher risk of cardiac events (syncope, aborted cardiac arrest) and that, in prepubertal children (<14 years old), girls had an equal likelihood of cardiac events as in boys.\(^{10} \) However, a closer scrutiny of the data revealed that boys had a 3-fold greater likelihood of a lethal arrhythmia.\(^{10} \) Thus, the lethality of LQT2 arrhythmias in boys trumps the number of cardiac events and indicates that the arrhythmia phenotype is reversed in children compared with adults. Thus, the arrhythmia phenotype found in adult and prepubertal rabbit hearts with drug-induced LQT2 are congruent with that found for LQT2 in humans.

Interestingly, APDs were longer in prepubertal female than male rabbits, yet E4031 elicited TdP within minutes in male hearts but merely prolonged APDs in female hearts.\(^{11} \) Thus, factors other than \( K^+ \) currents and APD prolongation must be considered to predict the arrhythmia phenotype; namely factors that the propensity to early afterdepolarizations (EADs).

The L-type \( Ca^{2+} \) channel is a major regulator of cardiac \( Ca^{2+} \) homeostasis and has been implicated in the genesis of EADs and TdP.\(^{12} \) The classic hypothesis of EAD genesis suggests that they arise from reactivation of \( I_{\text{Ca,L}} \).\(^{13,14} \) Evidence for this mechanism has come from experimental reactivation of \( I_{\text{Ca,L}} \) with Bay K4864\(^{13} \) and a theoretical model.\(^{15} \) Another hypothesis of EAD formation proposes that APD prolongation promotes cellular \( Ca^{2+} \) overload, triggering spontaneous \( Ca^{2+} \) release from the sarcoplasmic reticulum (SR),\(^{16} \) enhancing the turnover rate of the \( Na^+/Ca^{2+} \) exchanger (NCX) and its depolarizing current, \( I_{\text{NCX}} \).\(^{12,17,18} \) which may reactivate \( I_{\text{Ca,L}} \). In the classic hypothesis, the EAD voltage depolarization precedes the rise of intracellular free \( Ca^{2+} \), \([Ca^{2+}]_r \), whereas \([Ca^{2+}]_e \) precedes EADs in the alternative mechanism. Compelling support for the second hypothesis comes from simultaneous maps of APs and \([Ca^{2+}]_e \), in which E4031-induced EADs generated a rise of \([Ca^{2+}]_e \), of such magnitude and kinetics that it was most likely produced by spontaneous SR \( Ca^{2+} \) release.\(^{17} \) Nevertheless, both mechanisms implicate \( I_{\text{Ca,L}} \) as a trigger of EADs.

Studies of the genomic effects of estrogen on the expression of cardiac \( Ca^{2+} \) channels and \( I_{\text{Ca,L}} \) have yielded contradictory results. In papillary muscles of female rabbits, ovariec-tomy increased and estrogen replacement (7 days) decreased isometric force. Estrogen reduced \(^1H\)-nitrendipine binding in plasma membrane preparations compared with ovariectomy and control groups, yet peak L-type calcium currents (\( I_{\text{Ca,L}} \)) was not significantly different for the 3 treatment groups.\(^{19} \) In contrast, Pham et al reported higher \( I_{\text{Ca,L}} \) density on the epicardium of adult female rabbit hearts compared with males and no sex differences on the endocardium such that female hearts, but the authors did not examine apex–base differences in \( I_{\text{Ca,L}} \).\(^{20} \) In rat hearts, Western blots indicated that females had higher levels of ryano dine receptor, \( Ca_{1.2} \) (the \( \alpha \) subunit of the L-type \( Ca^{2+} \) channel protein), and NCX proteins, yet their mRNA levels were lower than males.\(^{21} \)

New Zealand rabbits offer significant advantages as a model of human LQT2 and to investigate sex differences in arrhythmia phenotype. (1) Rabbit cardiac APs and ionic currents (in particular \( K^+ \) currents: \( I_{\text{Kr}}, I_{\text{Ks}}, I_{\text{K1}}, \) and \( I_{\text{K2}} \)) are similar to human APs, with similar responses to blockers of \( K^+ \) currents.\(^{22,23} \) (2) Sex differences in arrhythmia phenotype are similar in rabbits and men.\(^{11} \) (3) Numerous studies have used rabbit models of drug-induced LQT to investigate the factors that precipitate TdP.\(^{24–26} \) (4) Rabbits are “reflex ovulators” with estrogen levels that remain elevated until mating,\(^{27} \) which avoids estrogen oscillations that occur in most mammals during the estrus cycle and thereby minimizes estrogen-dependent genomic variations of ion channel expression.

Here, we investigated sex, age, and regional differences in voltage-gated \( Ca^{2+} \) channels by measuring \( I_{\text{Ca,L}} \) density using the whole-cell voltage-clamp technique; by analyzing \( Ca_{1.2} \) protein levels using Western blots; by analyzing mRNA levels using real-time PCR; by correlating the regional elevation of \( I_{\text{Ca,L}} \) to the origin of the earliest EADs and to the LQT2 arrhythmia phenotype by optical mapping; and by showing that adult female and prepubertal male myocytes were more prone to fire EADs using experimental and simulation techniques. These findings provide new insight on the mechanisms underlying the firing of EADs and on sex and age differences in arrhythmia phenotype in LQT2.

Materials and Methods

Arrhythmia Phenotype in Langendorff Model of Drug-Induced LQT2

New Zealand White rabbits were anesthetized with pentobarbital (50 mg/kg) and injected with heparin (200 U/kg IV). Hearts were excised and perfused in a Langendorff apparatus with a Tyrode’s solution containing (in mmol/L): 130 NaCl, 24 NaHCO\(_3\), 1.0 MgCl\(_2\), 1.2 NaHPO\(_4\), 4.0 KCl, 50 dextrose, 1.25 CaCl\(_2\) gassed with 95% O\(_2\) and 5% CO\(_2\) (pH 7.4). Perfusion pressure was adjusted to 60 to 70 mm Hg by controlling the flow rate of the perfusion. Hearts were placed in a specially designed chamber to reduce movement artifacts and control the temperature in the medium bathing the heart using a feedback control device to maintain temperature at 37.0±0.2°C.\(^{28} \) Hearts were stained with the voltage-sensitive dye di-4-ANEPPS (25 \( \mu \)L of 1 mg/mL DMSO) (Molecular Probes, Eugene, Ore) by injecting the dye through a port in the bubble trap (or a compliance chamber) located above the aortic cannula to the heart.\(^{18} \) Hearts were then perfused with the \( I_{\text{Kr}} \) blocking agent E4031 (1-{[(1-{(6-methyl-2-pyridyl})-ethyl]-4-(4- methylsulfonylaminobenzoyl}) piperidine; 0.5 \( \mu \)mol/L) to produce a drug-induced LQT2 and allowed to beat at their intrinsic rate, as previously described.\(^{11} \) Four groups of rabbits were tested: (1) adult males (n=8) and (2) adult females (n=8) 3 to 4 month old; and (3) prepubertal males (n=10) and (4) prepubertal females (n=18) 6 weeks old weighing ~1.5 kg. For each Langendorff heart, the arrhythmia phenotype was determined by treating the heart with E4031 and tracking the emergence of EADs and TdP.
which typically occurred within 5 minutes or failed to occur for more than 30 minutes. Thus, the protocol using E4041 at 0.5 \text{ mmol/L} provided “yes” or “no” assay of arrhythmia phenotype.

**Regional Distribution of the Earliest EADs**

On perfusion with E4031, APs and EADs were monitored by optical mapping to identify the locations on the heart that fired the earliest EADs that progressed to TdP. In cases where EADs appeared at several sites on the epicardium, the earliest EAD was identified from the temporal delays between all sites that fired an EAD. Precautions were taken to ensure that EAD signals represented electric events and not motion artifacts. The earliest EAD had to occur synchronously with a voltage change measured by interface EKG recordings and had to propagate to adjacent regions of the heart for at least \( \approx 3 \text{ mm} \) or 3 pixels. In most cases, the first EADs appeared at the base exclusively and propagated out but did not reach the apex regions. In other cases, EADs appeared first at the base and propagated to the apex; in those cases, an activation map was generated to ascertain the origin of the EAD wave. Cumulative plots of the sites that fired the earliest EADs were generated separately for adult female and prepubertal male rabbit hearts to determine whether EADs were more likely to start from basal or apical regions of the ventricles. A 1-tailed “binomial test” was used as a test for the statistical significance of deviation from the null hypothesis (\( P=0.5 \), or 50% probability) that EADs were equally likely to occur at the base or the apex. Statistical significance for EADs firing from a preferential location is reached when the binomial test rejects the null hypothesis with \( P \leq 0.05 \). The probability of firing the earliest EAD at the apex is given by:

\[
P = \Sigma(C_n) \times (1/2)^n
\]

where the sum is taken from \( j=0 \) to \( a \), \( a \) is the number of experiments in which EADs fire first at the apex, \( N \) is the total number of experiments in which EADs were measured, and \( C_n \) is the combination of “a” out of “N.”

**Cell Isolation**

Ventricular myocytes were isolated from either prepubertal (30- to 49-day-old) or adult (3-month-old) male and female New Zealand White rabbits by a modification of a previously described method. Briefly, rabbits were anesthetized with pentobarbital (50 mg/kg) and injected with heparin (200 U/kg IV). The hearts were excised and perfused via the aorta with a physiological salt solution (PSS) containing (in mmol/L): 140 NaCl, 5.4 KCl, 1.5 CaCl\(_2\), 2.5 MgCl\(_2\), 11 glucose, and 5.5 HEPES (pH 7.4). Hearts were then perfused with Ca\(^{2+}\)-containing PSS for 5 minutes, followed by perfusion with nominally Ca\(^{2+}\)-free PSS for 10 minutes, after which collagenase type 2 (Worthington; at 0.60 mg/mL) was added to Ca\(^{2+}\)-free PSS for 15 minutes of digestion at 35°C. The ventricles were removed and placed in a high potassium buffer containing (in mmol/L): 110 K-glutamate, 10 KH\(_2\)PO\(_4\), 25 KCl, 2 MgSO\(_4\), 20 taurine, 5 creatine, 0.5 EGTA, 20 glucose, and 5 HEPES (pH 7.4). Sections of the epicardium approximately 1 mm in depth were surgically removed from the apex and base regions of the left ventricle, and cell isolation was performed separately for each region. Myocytes from the apex were taken from 3 to 6 mm from the very bottom of the heart, those from the base were taken from 1 to 4 mm below the left atrium, and no cells were studied from a 3 to 4 mm region in the middle of the heart. The tissues were minced, and the single myocytes were obtained by filtering through a 100-\( \mu \)m nylon mesh. Cells were allowed to settle, the supernatant was aspirated, and the pellet was resuspended in high potassium buffer. Experiments were performed on the day of cell isolation and 4 to 8 myocytes were studied from each heart.

The methods and protocols used in the study were all in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals as adopted by the NIH.

**Data Acquisition and Analysis**

L-type Ca\(^{2+}\) currents were studied using the conventional whole-cell configuration of the patch clamp technique. Patch pipettes had resistances of 1 to 2.5 M\( \Omega \) when filled with (in mmol/L): 130 CsCl, 20 tetraethylammonium chloride (TEA-Cl), 5 MgATP, 5 EGTA, 0.1 Tris-GTP, and 5 HEPES (pH 7.2). Cells were bathed in K\(^{-}\)-free solution containing (in mmol/L): 140 NaCl, 5.4 CsCl, 2.5 CaCl\(_2\), 0.5 MgCl\(_2\), 11 glucose, and 5.5 HEPES (pH 7.4). Currents recorded using an Axopatch 200 amplifier were filtered at 5 kHz and sampled at 10 kHz using a Digidata 1200a interface and pCLAMP (version 9.2) software (Axon Instruments). The magnitude of the peak inward current \( I_{\text{Ca,L}} \) was measured during 100-ms voltage-clamp steps to 0 mV applied following a 50-ms prepulse to −30 mV from a holding potential of −80 mV every 6 seconds. All recordings were made 3 to 5 minutes after gaining whole-cell access and after \( I_{\text{Ca,L}} \) had stabilized. Series resistance was partially compensated to achieve values of ≤3.0 m\( \Omega \) to prevent large voltage errors when measuring larger (1-5 nA) whole-cell \( I_{\text{Ca,L}} \). Capacitance measurements were obtained from membrane test parameters using Axon software. Capacitance of male and female prepubertal myocytes was 67.2±2.1 and 69.9±3.7 pF, respectively. Adult male and female myocyte capacitance was 110.7±7.3 and 109.3±8.7 pF, respectively. \( I_{\text{Ca,L}} \) was isolated by blocking K\(^{+}\) channels with Cs\(^{+}\) and TEA-Cl, inactivating Na\(^{+}\) channels with a voltage-clamp prepulse step to −30 mV and eliminating the driving force for Cl\(^{−}\) currents by measuring \( I_{\text{Cl,L}} \) close to the predicted Cl\(^{−}\) equilibrium potential (0 mV).

The voltage dependence of Ca\(^{2+}\) channel activation and inactivation was determined as described previously. Parameters for the voltage dependence of activation were obtained from the least squares fit of data points to the equation:

\[
g_{\text{Ca,act}} = I / [(1 + \exp(-(V_{m}-V_{1/2,b})/b))]
\]

where \( g_{\text{Ca,act}} \) represents normalized Ca\(^{2+}\) conductance, \( V_{m} \) represents test potentials from −30 to 30 mV, \( V_{1/2,b} \) is the potential at half-maximal activation, and \( b \) is the slope. Parameters for the voltage dependence of inactivation were obtained from the equation:

\[
I = I_{0} + (1-I_{0})/[1 + \exp(-(V_{m}-V_{1/2,a})/b)]
\]

where \( I_{0} \) is the normalized magnitude of the peak inward current measured during a test pulse to 0 mV following a 5-second conditioning pulse (\( V_{c} \)) to \( V_{m} \) between −90 and 30 mV, \( L_{0} \) is the inactivation-resistant current, \( V_{1/2,a} \) is the potential at which inactivation was half maximal, and \( b \) is the slope. The current elicited during the test pulse was normalized to the magnitude of the current recorded during a pretest pulse to 0 mV, which preceded each conditioning pulse. This corrected for changes in current magnitude attributable to half-maximal inactivation of \( I_{\text{Ca,L}} \) (\( I_{0} \)) was determined by fitting the inactivating component of the \( I_{\text{Ca,L}} \) trace (defined as the region between the peak Ca\(^{2+}\) current and the end of the depolarizing pulse to 0 mV) to the biexponential curve fitting function of Clampfit (Axon Instruments). The larger of the 2 exponential components (97% of the inactivation curve) was used to measure \( V_{1/2,a} \). Results are reported as the mean±SE of at least 3 or more independent experiments. Statistical comparisons between 2 groups of experimental data were performed using the Student’s 2-tailed t test.

**Action Potentials**

APs were recorded using the current-clamp mode as previously described with an internal solution containing (in mmol/L): 150 KCl, 5 MgATP, 5 EGTA, 0.1 Tris-GTP, and 5 HEPES (pH 7.2). Cells were bathed in an extracellular solution containing (in mmol/L): 140 NaCl, 5.4 KCl, 2.5 CaCl\(_2\), 0.5 MgCl\(_2\), 11 glucose, and 5.5 HEPES (pH 7.4), at 35°C. APs were elicited by injecting current pulses (1 to 2 nA) of 5-ms duration through the patch pipette at frequencies of 0.33 or 1 Hz. Once stable, AP recordings were obtained, myocytes were exposed to 5 mmol/L E4031 to completely block \( I_{\text{Ca,L}} \) and recordings were continued to monitor AP prolongation and the incidence of EADs. Fisher’s exact test was used to test the null hypothesis of equal probability (\( P \)) of EADs between male

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Table 1. Parameters of Voltage-Dependent $\text{I}_\text{Ca,L}$ Activation and Inactivation in Prepubertal Myocytes

<table>
<thead>
<tr>
<th>Prepubertal</th>
<th>$V_\text{r}$ (mV)</th>
<th>$V_{1/2}$ (mV)</th>
<th>Slope</th>
<th>n</th>
<th>$V_{0.5,\text{inact}}$ (mV)</th>
<th>Slope</th>
<th>$\zeta$ (%)</th>
<th>n</th>
<th>$t_{1/2}$ (ms)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Apex</td>
<td>54±2.0</td>
<td>-4.7±0.8</td>
<td>7.9±0.8†</td>
<td>30</td>
<td>30±0.5*</td>
<td>-5.3±0.2</td>
<td>5.7±1.0</td>
<td>6</td>
<td>24±0.6‡</td>
<td>30</td>
</tr>
<tr>
<td>Base</td>
<td>55±2.1</td>
<td>-4.6±2.2</td>
<td>7.1±0.7‡</td>
<td>25</td>
<td>29±0.6</td>
<td>-5.3±0.2</td>
<td>4.1±0.9</td>
<td>7</td>
<td>21±0.5‡</td>
<td>25</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Apex</td>
<td>55±1.8</td>
<td>-5.3±2.3</td>
<td>8.0±0.7**</td>
<td>18</td>
<td>-33±0.6*</td>
<td>-5.9±0.1</td>
<td>4.6±0.5</td>
<td>7</td>
<td>22±0.6§</td>
<td>18</td>
</tr>
<tr>
<td>Base</td>
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<td>-5.4±2.7</td>
<td>7.4±0.4**</td>
<td>17</td>
<td>-32±0.6</td>
<td>-5.8±0.2</td>
<td>5.0±0.4</td>
<td>7</td>
<td>22±0.7</td>
<td>17</td>
</tr>
</tbody>
</table>

Cumulative data were analyzed as in Materials and Methods and expressed as means±SE. $V_\text{r}$ indicates reversal potential; $\zeta$, inactivation resistant current. *The voltage of half-maximal inactivation ($V_{0.5,\text{inact}}$) was significantly shifted to hyperpolarized potentials in female compared with male myocytes ($P<0.003$); †the slope factor in male† and female** apex myocytes was significantly different from base myocytes ($P<0.001$); ‡the time to half inactivation of $\text{I}_\text{Ca,L}$ ($t_{1/2}$) was significantly different between male apex and base ($P<0.0001$); $\S$male and female apex (P<0.05); n, number of cells in each group.

and female myocytes. A probability of <2% ($P<0.02$) is considered to be a rejection of the null hypothesis and indicates a statistically significant difference in the likelihood of firing an EAD between male and female myocytes.

Quantitative Assays of Protein and mRNA

Tissues from the apex or base of rabbit hearts were dissected from exactly the same regions as described for the isolation of ventricular myocytes for voltage-clamp studies. The tissues were disrupted using a PowerGen model 125 homogenizer (setting 5, 30 seconds) in 1 mL enhanced lysis buffer (ELB) (250 mmol/L NaCl, 0.1% NP-40, 50 mmol/L HEPES [pH 7.0], 5 mmol/L EDTA, 0.5 mmol/L dithiothreitol) supplemented with a cocktail of protease and phosphatase inhibitors. The extract was rocked for 15 minutes at 4°C, cellular debris was removed by centrifugation (12 000 g, 5 minutes), and supernatant containing the protein extract was stored at −80°C. Protein concentrations were determined by the Bradford method (Bio-Rad protein assay). Cell lysates (50 μg protein/lane) were fractionated by SDS-PAGE, transferred to poly(vinylidene difluoride) membranes (Immobilon-P, Bedford, Mass), and incubated overnight at 4°C with a rabbit antibody (Affinity Bioreagents, Golden, Colo) that was directed against Ca$_2^+$ (1.2) (diluted 1:1000) or mouse β-actin (Sigma, St Louis, Mo; diluted 1:20 000), followed by horseradish peroxidase–conjugated second antibody (Sigma). The antigen–antibody complex was visualized with Millipore Immobilon Western Chemiluminescent horseradish peroxidase substrate. Digitized fluorograms were quantified by using NIH Image 1.6 Software.

mRNA Extraction and Real-Time PCR

Left ventricular epicardial tissue from the apex or base of rabbit hearts was disrupted as described for protein extracts but in guanidine thiocyanate buffer, and mRNA was purified by centrifugation through cesium chloride. The resulting RNA (200 ng) was subjected to reverse transcription in 100 μL of Geneamp PCR buffer (Applied Biosystems) containing 1 mmol/L dNTPs, 2.25 mmol/L random hexamers, 7.5 mmol/L MgCl$_2$, and Superscript II (Invitrogen). The reaction was carried out in a thermocycler at 25°C for 5 minutes, 48°C for 30 minutes, and 95°C for 5 minutes. The real-time PCR was performed using 2 μL of the reverse transcription reaction products in a total volume of 50 μL containing 25 μL of Absolute SYBR Green mix (Thermo Scientific) and 20 mmol/L primers. The reaction was carried out in an Applied Biosystems 7900HT thermocycler. Ca$_2^+$ (1.2) expression was normalized to that of GAPDH. The primers used included the following: Ca$_2^+$ (1.2) forward, 5′-CATTTGGAACATGTGATTGTC-3′; Ca$_2^+$ (1.2) reverse, 5′-CAGGCGAAATGAACTGCAG-3′; GAPDH forward, 5′-TCCTGGGCTACACCGAGG-3′; and GAPDH reverse, 5′-GGCCACTGTGGAACTCCAG-3′. Relative quantitation was carried out using the 2$^{-\Delta\Delta Ct}$ method.

Action Potential Modeling of Cardiac Myocytes

Left ventricular epicardial APs were simulated using the Luo–Rudy (LRd) model of the mammalian ventricular AP.$^{35,36}$ The model incorporates the transient outward K$^+$ current, $\text{I}_\text{Kr}$, and ionic currents are mathematically represented by the Hodgkin–Huxley formulation. The computational model includes simulations for ion transporters and pumps that regulate Na$^+$ and Ca$^{2+}$ movements in the myocardium. The model tracks the dynamic changes in intracellular Ca$^{2+}$ by incorporating Ca$^{2+}$ release and uptake from the SR network, a delay for Ca$^{2+}$ diffusion from the longitudinal to junctional SR, NCX, and Ca$^{2+}$ buffering by calmodulin, troponin (in the cytoplasm), and calsequestrin (in the SR). Experimentally determined sex, age, and regional differences in current densities and voltage-dependent parameters for $\text{I}_\text{Ca,L}$ (Tables 1 and 2) were incorporated into the AP model by modifying the

Table 2. Parameters of Voltage-Dependent $\text{I}_\text{Ca,L}$ Activation and Inactivation in Adult Myocytes

<table>
<thead>
<tr>
<th>Adult</th>
<th>$V_\text{r}$ (mV)</th>
<th>$V_{1/2}$ (mV)</th>
<th>Slope</th>
<th>n</th>
<th>$V_{0.5,\text{inact}}$ (mV)</th>
<th>Slope</th>
<th>$\zeta$ (%)</th>
<th>n</th>
<th>$t_{1/2}$ (ms)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apex</td>
<td>55±1.2</td>
<td>-5.6±1.4</td>
<td>6.3±0.09</td>
<td>7</td>
<td>-29±1.20</td>
<td>-5.0±0.44</td>
<td>4.5±0.58</td>
<td>6</td>
<td>17.2±0.50*</td>
<td>7</td>
</tr>
<tr>
<td>Base</td>
<td>55±1.3</td>
<td>-4.6±1.1</td>
<td>6.3±0.09</td>
<td>10</td>
<td>-28±1.84</td>
<td>-5.6±0.16</td>
<td>3.3±0.58</td>
<td>6</td>
<td>17.3±0.64*</td>
<td>10</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apex</td>
<td>56±1.3</td>
<td>-4.6±0.78</td>
<td>6.7±0.18</td>
<td>9</td>
<td>-28±1.36</td>
<td>-5.3±0.30</td>
<td>5.7±0.75</td>
<td>5</td>
<td>18.8±1.1*</td>
<td>8</td>
</tr>
<tr>
<td>Base</td>
<td>54±0.75</td>
<td>-6.6±0.87</td>
<td>6.2±0.14</td>
<td>10</td>
<td>-26±1.95</td>
<td>-5.1±0.36</td>
<td>4.6±0.33</td>
<td>6</td>
<td>17.0±0.9*</td>
<td>11</td>
</tr>
</tbody>
</table>

Cumulative data were analyzed as in Materials and Methods and expressed as means±SE. $V_\text{r}$ indicates reversal potential; $V_{1/2}$, the voltage that gave half-maximal activation or inactivation ($V_{0.5,\text{inact}}$); $\zeta$, inactivation resistant current. *Time to half inactivation of $\text{I}_\text{Ca,L}$ in adult myocytes was significantly different from corresponding sex and regions of prepubertal myocytes; n, number of cells in each group.
equations representing $I_{Ca,L}$. The online data supplement provides definitions of the simulations parameters used to model APs from the base of prepubertal adult and male and female myocytes. To mimic LQT2, the rapid component of the delayed rectifier K$^+$ current, $I_{Kr}$, was suppressed by either 50% or 100%. The last AP from a train of 50 APs with a simulated cycle length of 1 second displayed was used to evaluate the effects of altered $I_{Ca,L}$ on APs with suppressed $I_{Kr}$. Simulations were repeated to examine the influence of the NCX and its current, $I_{NCX}$, on APD and EAD generation during 50% or 100% $I_{Kr}$ block with and without an increase of $I_{Ca,L}$. The LRd model has been extensively used in several studies during the past decade to understand the mechanisms of arrhythmias arising from ion channel mutations and/or drug block.\(^{38–40}\)

**Results**

**Sex and Age Differences in Arrhythmia Phenotype**

Adult rabbit hearts exhibited the expected sex differences in arrhythmia phenotype as that reported for clinical drug-induced LQT2. As shown in Figure 1, perfusion of adult male hearts with E4031 produced a marked prolongation of APDs (>1 second) yet failed to develop TdP (1 of 8 hearts had an arrhythmia) (Figure 1a). In contrast, female hearts treated with the same concentration of E4031 consistently developed TdP in 7 of 8 hearts (Figure 1b). The arrhythmia phenotype was opposite in hearts isolated from prepubertal rabbits where perfusion with E4031 elicited TdP in male hearts (7 of 10 hearts, as in Figure 1c) but failed to elicit TdP in prepubertal female hearts (2 of 18 developed TdP, Figure 1d). Note the drug E4031 was effective in all cases and caused a marked APD prolongation in adult males and prepubertal female hearts yet no TdP. The highly reproducible sex differences of arrhythmia phenotype in adults and the reverse in prepuberty did not correlate with sex differences in APDs where prepubertal female hearts had longer APDs than their male counterparts.\(^{11}\)

**Sex and Regional Comparisons of Epicardial $I_{Ca,L}$ in Prepubertal Rabbit Hearts**

In prepubertal hearts, peak whole-cell Ca$^{2+}$ currents (normalized to cell capacitance) were significantly higher in male compared with female epicardial myocytes isolated from the base of the left ventricles (Figure 2). $I_{Ca,L}$ measured at 0 mV from the base of the heart was higher in absolute magnitude in male (−6.4 ± 0.5 pA/pF; cells [n]=26; hearts [H]=7) compared with female myocytes (−5.0 ± 0.2 pA/pF; n=17; H=4; P<0.03). Representative individual current traces were superimposed (Figure 2A) to demonstrate the differences in $I_{Ca,L}$ between the sexes. Current-to-voltage (I/V) relationships were plotted for test potentials between −30 and +60 mV (Figure 2B). I/V plots were bell-shaped for both sexes, reached a single maximum value at 10 mV, and had identical reversal potentials ($V_r$) (see Table 1).

At the apex, no significant differences were found in peak $I_{Ca,L}$ between prepubertal male (−5.0 ± 0.3 pA/pF; n=30; H=9; P<0.02) and female (−4.8 ± 0.3 pA/pF; n=18; H=5) myocytes. The superposition of current traces from male and female myocytes (Figure 2C) demonstrated that at the apex, $I_{Ca,L}$ was similar for both sexes. Averaged I/V relationships (Figure 2D) and cumulative data for $I_{Ca,L}$ measured at 0 mV (Figure 2E) demonstrated that the normalized current magnitudes were comparable in both sexes for myocytes isolated from the apex of the hearts.

The apex–base distribution of $I_{Ca,L}$ in prepubertal male and female ventricles were analyzed because previous reports implicated regional heterogeneities in current distribution as contributors to dispersion of repolarization and arrhythmia vulnerability.\(^{11,20,33}\) Individual current traces and I/V plots (Figure 2A through 2E) showed that male epicardial cells from the base had significantly higher peak $I_{Ca,L}$ (−6.4 ± 0.5 pA/pF) than those from the apex (−5.0 ± 0.3 pA/pF). In
female prepubertal rabbit hearts, apex–base differences in $I_{\text{Ca,L}}$ ($-4.8 \pm 0.3 \text{ nA/pF}$ versus $-5.0 \pm 0.2 \text{ nA/pF}$) were not statistically significant (Figure 2E).

**Voltage Dependence of $I_{\text{Ca,L}}$ in Prepubertal Rabbit Hearts**

The voltage dependence of $I_{\text{Ca,L}}$ activation and inactivation was measured to determine whether sex and regional differences exist in these channel properties. $I_{\text{Ca,L}}$ activation curves for male and female apex and base epicardial myocytes are presented in Figure 3A. Although sex differences in $I_{\text{Ca,L}}$ activation were not observed, there were significant regional differences in the slope factor for $I_{\text{Ca,L}}$ activation in both sexes (Table 1). In males, the slope factor for current activation at apex and base was $7.9 \pm 0.84$ and $7.1 \pm 0.67$, respectively ($P<0.001$). The slope factor for current activation measured in female apex and base myocytes was $8.0 \pm 0.67$ and $7.4 \pm 0.42$, respectively ($P<0.01$). The higher slope factor implied that during an AP the time course of Ca$^{2+}$ entry via voltage-gated Ca$^{2+}$ channels may be faster at the apex than the base of the heart.

As shown in Figure 3B, $I_{\text{Ca,L}}$ inactivation occurred at more negative potentials in female compared with male myocytes. In female apex and base epicardial myocytes, the voltage at half-maximal inactivation ($V_{1/2}$) was $-33 \pm 0.6$ and $-32 \pm 0.6 \text{ mV}$, respectively. The $V_{1/2}$ of current inactivation in male apex and base cells occurred at significantly more positive potentials of $-30 \pm 0.5$ and $-29 \pm 0.6 \text{ mV}$, respectively ($P<0.03$).

Analysis of $I_{\text{Ca,L}}$ inactivation kinetics revealed that the time to half-maximal inactivation ($t_{1/2}$) was significantly longer in male ($24.0 \pm 0.56 \text{ ms}$; $n=30$; $H=9$) compared with female ($22.2 \pm 0.58 \text{ ms}$; $n=18$; $H=5$; $P<0.05$) myocytes at the apex. No sex differences were observed at the base. In addition, there were marked apex–base differences in $t_{1/2}$ in male hearts; $t_{1/2}$ was significantly longer at the apex than the base ($24.0 \pm 0.56 \text{ vs. } 20.6 \pm 0.45 \text{ ms}$; $n=26$; $H=7$; $P<0.0001$), whereas regional differences in $t_{1/2}$ were not observed in female hearts.

**Sex and Regional Comparisons of $I_{\text{Ca,L}}$ in Adult Rabbits**

In contrast to the findings in prepubertal hearts, peak $I_{\text{Ca,L}}$ was significantly higher at the base of adult female compared with male myocytes. Representative current traces from myocytes with nearly identical membrane capacitance (Figure 4A), averaged I/V relationships (Figure 4B) and cumulative data (Figure 4E) demonstrated the
Significant differences in the rate of \(I_{\text{Ca,L}}\) inactivation were observed between adult and prepubertal hearts. The \(t_1/2\) for \(I_{\text{Ca,L}}\) at the base of adult hearts was 17.3±0.64 and 17.0±0.87 ms for male and females, respectively. The \(t_1/2\) values in male and female apex myocytes of adult hearts were 17.2±0.50 and 18.8±1.1 ms, respectively. These figures were statistically significant (\(P<0.01\)) for \(t_1/2\) values of prepubertal hearts (Tables 1 and 2).

**Sex, Age, and Regional Distribution of Ca,1.2\(\alpha\)**

A reasonable explanation for sex differences in \(I_{\text{Ca,L}}\) is that sex steroids modulate the expression levels of the \(Ca^{2+}\) channel protein Ca,1.2\(\alpha\). Quantitative Western blot analysis of total protein supported the notion that differences in the current density of \(I_{\text{Ca,L}}\) was attributable to differences in protein levels. Hearts from adult and prepubertal rabbits of each sex (\(n=4\) per group) were rapidly isolated and flash-frozen in liquid nitrogen, and segments of tissue were dissected for protein and mRNA analysis. The tissues were dissected from exactly the same regions of epicardium as described for the isolation of myocytes for \(I_{\text{Ca,L}}\). In adult rabbits, Ca,1.2\(\alpha\) was expressed at a statistically significant higher level at the base of female hearts compared with the apex and was higher than at the base and apex of male hearts (Figure 6a and 6c). In prepubertal rabbits, Ca,1.2\(\alpha\) was higher at the base of male hearts compared with the apex and was higher than at the base and apex of female hearts, but the differences were not statistically significant (Figure 6b and 6d). Ca,1.2\(\alpha\) mRNA levels exposed statistically significant higher levels at the base than apex of adult female hearts but were otherwise not statistically significant in either adult or prepubertal comparisons of mRNA levels (Figure 6e and 6f).

**Spatial Distribution of the Earliest EADs**

The correlation between the arrhythmia phenotype and the enhanced \(I_{\text{Ca,L}}\) at the base of the hearts suggests that the earliest EADs that capture and progress to TdP should also occur at the base of adult female and prepubertal male hearts. More precisely, a higher \(Ca^{2+}\) current density was measured from myocytes isolated from the top one-third of the base, just below the left atrium, compared with the bottom one-third of the apex.

To measure the spatial distribution of the earliest EADs on the epicardium, E4031 was added to the perfusate and maps of optical APs were recorded to detect the earliest EADs that appeared on the epicardium. As shown in Figure 7, the location of the earliest EADs clustered around the base of the heart in both adult female (Figure 7A; \(n=9/9\) hearts) and prepubertal male hearts (Figure 7B; \(n=8/9\) hearts). For these measurements, only hearts that developed E4031-induced TdP were considered in the analysis, and only 1 prepubertal male heart had an early EAD that occurred below the midline (red horizontal line, Figure 7A and 7B). A 1-tailed binomial test was used to test the hypothesis that EADs occur with equal probability at the base and the apex. For adult female and prepubertal males, the probability values were 0.001953 and 0.017578, respectively. Thus, the clustering of EADs around the base of the heart was statistically significant, with \(P<0.002\).

**Sex Differences on the Incidence of EADs in Isolated Myocytes**

APs were recorded from ventricular myocytes isolated from the base of prepubertal and adult male and female hearts;
then, treatment with E4031 revealed a sex-dependent propensity to fire EADs. As shown in Figure 8, prepubertal male (Figure 8A; n=4 cells; H=3 hearts) and adult female (Figure 8D; n=6; H=4) myocytes fired EADs, whereas prepubertal female (Figure 8B; n=4; H=3) and adult male (Figure 8C; n=6; H=4) myocytes failed to fire EADs when treated with E4031. In all myocytes, E4031 produced a marked APD prolongation, but EADs could only be observed in prepubertal male and adult female myocytes. To observe EADs, the external Ca\(^{2+}\) concentration had to be raised to 2.5 mmol/L and the myocytes had to be paced for 10 to 20 beats at 1 Hz for prepubertal myocytes and at 0.33 Hz for adult myocytes. Fisher’s exact test was applied to test the null hypothesis that both males and females have equal likelihood of having EADs. The null hypothesis is rejected because of its probability (P=0.0143 and 0.0076 for prepubertal and adult myocytes, respectively). With P<0.02, statistical significance is achieved for the greater incidence of EADs in prepubertal males compared with female and for adult female compared with adult male myocytes.

**Figure 4.** Sex differences in I\(_{Ca,L}\) are reversed in adult rabbits. A, Representative I\(_{Ca,L}\) traces from adult female and male base myocytes with similar sizes or capacitance (±3 pA/pF). B, I/V relationships from adult female and male base myocytes. C, Representative traces from female and male base myocytes of similar capacitance (±3 pA/pF). D, I/V relationships in female and male apex myocytes. E, Cumulative differences in peak I\(_{Ca,L}\) between female and male base myocytes. The magnitude of peak I\(_{Ca,L}\) between sexes was significantly different at the base (n=9 to 11; P<0.01) but not at the apex. ns indicates not significantly different (n=7 to 9; P<0.50).

**AP Simulations: Influence of Elevated I\(_{Ca,L}\) on EAD Induction**

Simulations of the cardiac AP based on a modified version of the LRd model were used to evaluate the role of enhanced I\(_{Ca,L}\) density as a predictor of the propensity to EADs in drug-induced LQT2. AP simulations for prepubertal and adult myocytes from the base of the heart were generated by incorporating the experimentally determined current densities and voltage-dependent parameters for I\(_{Ca,L}\) (Figures 2 through 5 and Tables 1 and 2) by modifying the equations represent-
ing $I_{Ca,L}$. To mimic LQT2, the rapid component of the delayed rectifier K$^+$ current, $I_K$, was suppressed by either 50% or 100%. Figure 9a and 9b illustrates simulations of control APs in prepubertal male and female myocytes, respectively. Although experimental differences in $I_{Ca,L}$ properties (see Table 1) were incorporated in the simulations of control APs, there were no discernable differences in the shape and time course of prepubertal APs. However, the subsequent 50% block of $I_K$ resulted in the firing of EADs in the male (higher $I_{Ca,L}$) but not the female model of a myocyte. In adults, female and male myocytes were modeled according experimental differences in their $I_{Ca,L}$ properties (Table 2), and, again, there were no discernable differences in control APs (Figure 9c and 9d). When a 50% inhibition of $I_K$ was inserted, female myocytes fired EADs, whereas male myocytes did not (Figure 9c and 9d). The theoretical analysis confirmed the experimentally recorded APs (Figure 8) using E4031 to suppress $I_K$ and mimic the propensity to fire EADs, which, in turn, are consistent with the arrhythmia phenotype recorded in intact perfused hearts. The simulations support the hypothesis that a 25% to 30% increase of $I_{Ca,L}$ was alone sufficient to promote EADs in myocytes with reduced $I_K$.

**Influence of $I_{NCX}$**

A reasonable concern is that the upregulation of $I_{Ca,L}$ will increase Ca$^{2+}$ influx on a beat-to-beat basis, which is likely to be accompanied by an increase in $I_{NCX}$ to increase Ca$^{2+}$ efflux and balance influx to efflux. Based on simulations of the AP, Figure 10 shows that in prepubertal male (Figure 10A) and adult female (Figure 10B) myocytes, an increase in $I_{NCX}$ (30%) had no discernible effect on APD and that during 100% or 50% $I_K$ block, the higher $I_{NCX}$ does not inhibit the generation of EADs. Nevertheless, a 30% increase in $I_{NCX}$ decreased intracellular free Ca$^{2+}$ in the cytosol (Figure 10D) and the Ca$^{2+}$ concentration in the lumen of the SR (Figure 10E). Moreover, during $I_K$ blockade, increasing $I_{NCX}$ alone did not elicit EADs (Figure 10C), whereas increasing $I_{Ca,L}$ alone was sufficient to elicit EADs (Figure 9), which highlights the importance of $I_{Ca,L}$ as an important determinant of EADs susceptibility and of arrhythmia phenotype.

**Discussion**

Our main findings are that $I_{Ca,L}$ is elevated at the base of hearts that are prone to EADs and TdP in E4031-induced LQT2 and that the first EADs that elicit TdP originate from the base of the heart. Protein and mRNA levels suggest that these sex differences in $I_{Ca,L}$ are predominantly attributable to sex differences in ion channel expression at the base but not the apex of the left ventricle. More precisely, we found that $I_{Ca,L}$ at the base is higher in prepubertal male than female myocytes and that this sex difference is unique to the base of the heart and not the apex, resulting in a gradient of $I_{Ca,L}$ in prepubertal male but not in female hearts. Moreover, sex differences in $I_{Ca,L}$ are reversed in adult rabbit hearts such that the adult female myocytes now have higher $I_{Ca,L}$ at the base compared with adult males. I/V relationships and the kinetics of $I_{Ca,L}$ for all 4 groups of rabbits suggest that these sex differences in $I_{Ca,L}$ are attributable to genomic changes in the density of functional L-type channels rather than alterations in channel properties. Besides changes in peak $I_{Ca,L}$, differences in the $V_{0.5}$ suggest that inactivation of $I_{Ca,L}$ occurs later during the AP of adult female myocytes, which would tend to contribute to a slight increase in APD and higher Ca$^{2+}$ influx per AP. The other statistically different parameter was the slower inactivation kinetics of apical versus basal myocytes from prepubertal male hearts. The physiological consequences of slower inactivation remain unclear but suggest that if all else remains unchanged, then the Ca$^{2+}$-dependent inactivation of $I_{Ca,L}$ is delayed at the apex, perhaps increasing Ca$^{2+}$ influx during the AP plateau. Western blots of the Ca$^{2+}$-binding subunit of Ca$^{2+}$-channel proteins revealed that protein levels are higher at the base of adult female hearts compared with the apex of female hearts and are higher than protein levels at the base and apex of adult male hearts. In adult hearts, differences in Ca$^{2+}$ were statistically significant and were consistent with functional measurements of whole-cell current densities. The mRNA coding for Ca$^{2+}$ were statistically (2.5±2) higher at the base than the apex of female hearts, but there were no other differences in mRNA between male and female hearts. Thus, the whole-cell current, protein, and mRNA analyses support the interpretation that there are sex differences in the expression of voltage-gated
L-type Ca\textsuperscript{2+} channels at the base but not the apex of the heart. In prepubertal hearts, Cav1.2 protein levels were statistically higher at the base of male hearts compared with the apex and had a tendency to be greater than at the base and apex of female hearts without reaching statistical significance. Similarly, message levels for Cav1.2 were not statistically different in prepubertal hearts.

The correlation of the arrhythmia phenotype with (1) higher \(I_{Ca,L}\), (2) higher protein levels, (3) the firing of EADs in single cells and in simulations, and (4) a statistically higher incidence of EADs that originate first at the base of the heart, together, provide compelling evidence that \(I_{Ca,L}\) is an important determinant of the arrhythmia phenotype. A higher \(I_{Ca,L}\) reduces the repolarization reserve and during \(I_{Kr}\) inhibition can promote EADs by 1 of 2 possible mechanisms: (1) spontaneous reactivation of \(I_{Ca,L}\) during the long AP plateau or (2) the reactivation of \(I_{Ca,L}\) triggered by an inward \(I_{NCX}\), which is, in turn, elicited by spontaneous SR Ca\textsuperscript{2+} release. A 20% to
30% increase in $I_{Ca,L}$ is sufficiently large to enhance (1) Ca$^{2+}$ influx per AP and intracellular Ca$^{2+}$ load, (2) luminal Ca$^{2+}$ in the SR, (3) spontaneous SR Ca$^{2+}$ release and $I_{NCX}$ during $I_{Kr}$ inhibition, and (4) thus initiate EADs that progress to TdP. Optical mapping of membrane potential changes showed that in adult female hearts treated with E4031, EADs originated preferentially from the base. Similarly, AP recordings from adult ventricular myocytes isolated from the base of the heart showed that $I_{Kr}$ blockade with E4031 elicited EADs in female but not in male myocytes. Thus, single-cell properties are consistent with the arrhythmia phenotype of intact hearts. AP simulations confirmed that $I_{Kr}$ inhibition prolonged APDs without eliciting EADs and that an elevation of $I_{Ca,L}$ was necessary and sufficient to elicit EADs. These data do not exclude sex differences in the expression of other Ca$^{2+}$ channels and transporters, namely (1) $I_{NCX}$, (2) cardiac ryanodine receptor 2, and/or (3) SERCA2, SR Ca$^{2+}$ pumps.

**Sex Differences in $I_{Ca,L}$**

Several studies have investigated sex differences in $I_{Ca,L}$ in various mammalian species, but the findings remain inconclusive and no general consensus has thus far been achieved. In 50- to 60-day-old rabbits, Pham et al reported a transmural dispersion of $I_{Ca,L}$ (higher on the epicardium than endocardium) at the base of female hearts that was absent in male hearts. In contrast to rabbit hearts, a study on mongrel dogs found uniformly higher levels of $I_{Ca,L}$ in female than male hearts across the left ventricular wall. In guinea pig hearts, the opposite result was obtained, where $I_{Ca,L}$ was significantly higher in males than females even when the female current density was measured at different phases of the estrus cycle. Moreover, in mouse and rat hearts, no significant differences were found in $I_{Ca,L}$ between males and females. In human midmyocardial left ventricular myocytes from patients with end-stage heart failure, $I_{Ca,L}$ was found to be higher (~10%) in female than male hearts, but the difference did not reach statistical significance. Nevertheless, simulations and experiments showed that at long cycle lengths, myocytes from women were prone to EADs, whereas myocytes from men rarely fired an EAD. In the absence of similar studies in “healthy” human myocytes, Verkerk et al pointed out that the properties of myocytes from failing hearts were consistent with those obtained from healthy human hearts in terms of rate-corrected QT, EAD susceptibility, and sex differences in APDs and, thus, proposed that the differences in $I_{Ca,L}$ between female and male hearts represent a characteristic of normal human hearts.

Previous studies were attentive to transmural differences in $I_{Ca,L}$ but neglected apex–base differences or differences in...
pre- versus postpuberty. The current findings of higher $I_{\text{Ca,L}}$ in adult female base myocytes are in agreement with previous studies on rabbit and human hearts but extend the data to reveal $I_{\text{Ca,L}}$ differences between apex and base and between pre- and postpuberty. It may be that once apex–base heterogeneities are included in the analysis, $I_{\text{Ca,L}}$ differences between men and women will be statistically significant and will expose larger $I_{\text{Ca,L}}$ differences between the sexes. In rat and guinea pig hearts, sex differences in $I_{\text{Ca,L}}$ were not detected perhaps because the myocytes were isolated from random regions of the left ventricle, and regional heterogeneities of $I_{\text{Ca,L}}$ may conceal differences of 25% to 30% in current density. In another study, no apex–base differences in $I_{\text{Ca,L}}$ were detected in mongrel dogs and human hearts, but the study was not attentive to possible sex differences.

More intriguing is the finding that higher $I_{\text{Ca,L}}$ densities in adult and prepubertal rabbits correlate with the propensity to EADs at the base of the heart and the vulnerability to TdP in E4031-induced LQT2. Sex differences in arrhythmia phenotype (as defined by E4031 in perfused hearts) arise from the properties of ventricular myocytes because E4031 elicited EADs in freshly isolated adult female but not male myocytes.

Figure 8. EAD susceptibility in isolated ventricular myocytes from the base of the heart. Myocytes were isolated from the base of rabbit hearts as described in Materials and Methods and tested for their susceptibility to fire EADs once treated with E4031. In myocytes from prepubertal hearts, EADs occurred spontaneously in male (A) (n=4/4; H=3) but not in female (B) (n=0/4; H=1) ventricular cells. In myocytes isolated from adult hearts, there was a reversal of sex differences; EADs occurred in female (D) (n=3/8; H=4) but not in male (n=0/8; H=4) myocytes. Note that treatment with E4031 elicited a marked depolarization or EAD (Δ) in adult female and prepubertal male myocytes compared with their adult male and prepubertal female counterpart. Fisher’s exact test rejects the null hypothesis of equal probability of EADs between male and female myocytes with $P<0.02$, such that statistical significance is reached to predict that EADs are more likely to occur with prepubertal male than female myocytes and more likely to occur with adult female compared with male myocytes.

Regional Elevation of $I_{\text{Ca,L}}$ as a Predictor of Arrhythmia Phenotype

Numerous studies showed that enhanced dispersion of repolarization is proarrhythmic and contributes to the initiation of TdP induced by drugs that prolong the AP. In congenital, drug-induced LQT2 and in animal models of LQT2, APD prolongation does not automatically result in TdP and additional factors are needed to elicit EADs and TdP, with sex being a major risk factor. Our findings of higher $I_{\text{Ca,L}}$ at the base in pubertal males and adult females correlate with sex- and age-related differences in arrhythmia phenotype consistent with the hypothesis that, in the setting of prolonged APDs, the severity of Ca$^{2+}$ overload is a critical determinant of EADs and TdP. More precisely, the propensity to TdP in LQT2 is determined by $I_{\text{Ca,L}}$: the higher the current, the greater the severity of Ca$^{2+}$ overload and the greater propensity that EADs originate from the base and progress to TdP.

Study Limitations

A comprehensive analysis of the cellular determinants of LQT-mediated arrhythmias requires us to examine all chan-
reasonably expect that an increase in $I_{Ca,L}$ would be matched with an increase in Ca$^{2+}$ release channels at the cellular membrane and/or NCX to balance the influx to the efflux of Ca$^{2+}$ during an AP. Likewise, Ca$^{2+}$-handling proteins like the Ca$^{2+}$ release channels (or ryanodine receptors) and Ca$^{2+}$,Mg$^{2+}$-ATPase (SERCA2) on the SR network may exhibit sex differences and thereby contribute to the arrhythmia phenotype by altering SR Ca$^{2+}$ overload, spontaneous SR Ca$^{2+}$ release, and the initiation of EADs. In rat hearts, a recent study reported higher protein levels of Cav1.2, ryanodine receptor and NCX in females yet paradoxically Ca$^{2+}$,v1.2 mRNA levels were higher in males.\(^{21}\)

In adult and prepubertal rabbit hearts, we found sex differences in NCX protein that were similar to Ca$_{1.2a}$ with higher protein levels in adult female and prepubertal males based on Western blot analysis of prepubertal and adult rabbit left ventricles (not shown). Similarly, the density of $I_{NCX}$ was higher in prepubertal male compared with age-matched female base myocytes (not shown). Further studies that probe differences in Ca$^{2+}$ handling proteins are needed to obtain a better understanding of all determinants of the arrhythmia phenotype.

Optical mapping of membrane potential on the epicardium of rabbit hearts revealed a statistically significant propensity of EADs to appear first at the base of the heart. The 2D nature of optical mapping does not resolve the exact origins of such EADs but does identify the breakthrough sites that appear first on the epicardium. Until a high speed 3D technique is developed, one cannot completely exclude the possibility that EADs originate from deeper layers at the base of the ventricles. However, it is highly unlikely that EADs originate from deeper layers near the apex, which then propagate inside the ventricular wall to breakthrough at the base before the apex. The density of $I_{Ca,L}$ depends on the number of functional channels and on the modulation of channel activity by regulatory peptides and multiple phosphorylation sites through $\beta$-adrenergic activity. Sex-dependent regulation of channel activity presents another level of complexity that has yet to be analyzed in a comprehensive fashion. The findings raise important questions regarding genomic regulation of ion channel expression by sex steroids. What mechanisms produce sex differences in ion channel expression in prepuberty before the surge of estrogen and testosterone? What cues produce regional differences in ion channel expression?

Nevertheless, the role of $I_{Ca,L}$ as a determinant of arrhythmia phenotype in drug-induced LQT2 may be fundamental to our ability to evaluate the safety of new drugs that produce small but measurable QT or APD prolongation. Female sex is well known to be a risk factor to lethal Tdp, but the current data support the more precise notion that sex differences in $I_{Ca,L}$ is a critical factor in the assessment of arrhythmogenic risk. It is interesting to speculate that $I_{NCX}$ inhibition may pose less of an arrhythmogenic risk if it is combined with $I_{Ca,L}$ and/or $I_{NCX}$ inhibition. Similarly, congenital LQT2 may be asymptomatic well into adulthood; then, enhanced $I_{Ca,L}$ and/or...
I_{SCX} through a genomic regulation can precipitate a shift in arrhythmia phenotype. Thus, sex steroids, heart failure, and cardiac hypertrophy may alter the LQT2 arrhythmia phenotype through genomic regulation of Ca^{2+} channels.

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None.

References
41. Nakajima T, Hayashi K, Viswanathan PC, Kim MY, Anghelescu M, Barksdale KA, Shau W, Balser JR, Kupershmidt S. HERG is protected
from pharmacological block by alpha-1,2-glucosyltransferase function. 

differences in cardiac repolarization and ionic-current properties in canine

42. James AF, Arberry LA, Hancox JC. Gender-related differences in ven-
tricular myocyte repolarization in the guinea pig. *Basic Res Cardiol.*

43. Trepanier-Boulay V, St-Michel C, Tremblay A, Fiset C. Gender-based
2001;89:437–444.

44. Leblanc N, Chartier D, Gosselin H, Rouleau JL. Age and gender dif-

45. Verkerk AO, Wilders R, Veldkamp MW, de Geringel W, Kerkels JH, Tan
HL. Gender disparities in cardiac cellular electrophysiology and arrhyth-
mia susceptibility in human failing ventricular myocytes. *Int Heart J.*

46. Szentadrossy N, Banyasz T, Biró T, Szabo G, Toth BI, Magyar J, Lazar
J, Varro A, Kovacs L, Nanasi PP. Apico-basal inhomogeneity in distri-
bution of ion channels in canine and human ventricular myocardium.

47. Kanai A, Salama G. Optical mapping reveals that repolarization spreads
anisotropically and is guided by fiber orientation in guinea pig hearts.

48. Efimov IR, Huang DT, Rendt JM, Salama G. Optical mapping of repo-
larization and refractoriness from intact hearts. *Circulation.*

49. Liu DW, Gintant GA, Antzelevitch C. Ionic bases for electrophysio-
logical distinctions among epicardial, midmyocardial, and endocardial
myocytes from the free wall of the canine left ventricle. *Circ Res.*
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