

Azithromycin Causes a Novel Proarrhythmic Syndrome

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Background—The widely used macrolide antibiotic azithromycin increases risk of cardiovascular and sudden cardiac death, although the underlying mechanisms are unclear. Case reports, including the one we document here, demonstrate that azithromycin can cause rapid, polymorphic ventricular tachycardia in the absence of QT prolongation, indicating a novel proarrhythmic syndrome. We investigated the electrophysiological effects of azithromycin *in vivo* and *in vitro* using mice, cardiomyocytes, and human ion channels heterologously expressed in human embryonic kidney (HEK 293) and Chinese hamster ovary (CHO) cells.

Methods and Results—In conscious telemetered mice, acute intraperitoneal and oral administration of azithromycin caused effects consistent with multi-ion channel block, with significant sinus slowing and increased PR, QRS, QT, and QTc intervals, as seen with azithromycin overdose. Similarly, in HL-1 cardiomyocytes, the drug slowed sinus automaticity, reduced phase 0 upstroke slope, and prolonged action potential duration. Acute exposure to azithromycin reduced peak SCN5A currents in HEK cells ($IC_{50}=110\pm3\text{ }\mu\text{mol/L}$) and Na^+ current in mouse ventricular myocytes. However, with chronic (24 hour) exposure, azithromycin caused a ≈ 2 -fold increase in both peak and late SCN5A currents, with findings confirmed for I_{Na} in cardiomyocytes. Mild block occurred for K^+ currents representing I_{Kr} (CHO cells expressing hERG; $IC_{50}=219\pm21\text{ }\mu\text{mol/L}$) and I_{Ks} (CHO cells expressing KCNQ1+KCNE1; $IC_{50}=184\pm12\text{ }\mu\text{mol/L}$), whereas azithromycin suppressed L-type Ca^{++} currents (rabbit ventricular myocytes, $IC_{50}=66.5\pm4\text{ }\mu\text{mol/L}$) and I_{K1} (HEK cells expressing Kir2.1, $IC_{50}=44\pm3\text{ }\mu\text{mol/L}$).

Conclusions—Chronic exposure to azithromycin increases cardiac Na^+ current to promote intracellular Na^+ loading, providing a potential mechanistic basis for the novel form of proarrhythmia seen with this macrolide antibiotic. (*Circ Arrhythm Electrophysiol*. 2017;10:e003560. DOI: 10.1161/CIRCEP.115.003560.)

Key Words: calcium channel ■ mice ■ pharmacology ■ potassium channels ■ sodium channels

Sudden cardiac death caused by noncardiac medications is a major public health issue, in particular given its preventable nature. The scope of this problem is illustrated by the fact that drug-related proarrhythmia has been the most common cause for removal of medications from the US market in recent decades.¹ An improved understanding of the basic mechanisms causing drug-related sudden cardiac death would lead to safer pharmacotherapy.

See Editorial by Fishman

The macrolide antibiotics erythromycin and clarithromycin are associated with increased risk of serious ventricular tachyarrhythmias and sudden cardiac death, whereas azithromycin was long considered to have minimal adverse cardiac effects.² However, because of occasional case reports

of marked QT prolongation and serious ventricular arrhythmias, as well as concerning data from the US Food and Drug Administration Adverse Event Reporting System,^{3–10} we previously conducted a retrospective cohort study to examine azithromycin safety.¹¹ We found that azithromycin users had an increased risk of cardiovascular mortality and sudden cardiac death, compared with users of amoxicillin.

Several mechanisms have been described whereby drugs can increase susceptibility to serious ventricular tachyarrhythmias and sudden cardiac death, analogous to inherited arrhythmia syndromes. First, a reduction in cardiac Na^+ current (I_{Na}) can lead to slowed conduction, facilitating reentrant ventricular arrhythmias.^{12,13} This likely accounts for increased mortality in patients with coronary artery disease treated with Na^+ channel blockers and in the Brugada

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WHAT IS KNOWN

- The commonly used antibiotic azithromycin increases risk of cardiovascular and sudden cardiac death, but the underlying mechanisms are unclear.
- While torsades de pointes is rare, case reports indicate that azithromycin can also cause a novel proarrhythmic syndrome, characterized by polymorphic ventricular tachycardia in the setting of a normal QT interval.

WHAT THE STUDY ADDS

- Acutely, azithromycin causes diffuse depression of the cardiac conduction system and ion channel block, reminiscent of azithromycin overdose in humans.
- Chronically, the drug markedly potentiates cardiac sodium current to promote intracellular sodium loading, a condition associated with intracellular calcium overload and polymorphic ventricular tachycardia.
- These findings provide a unifying mechanistic basis for the novel form of proarrhythmia seen with azithromycin, which likely contributes to the increased risk of sudden cardiac death seen with this antibiotic.

syndrome, where Na^+ current is downregulated. In the setting of overdose and intravenous administration,^{14,15} azithromycin can cause QRS widening suggesting I_{Na} block, but this has not been reported during oral dosing. Second, excessive prolongation of ventricular repolarization promotes abnormal triggered activity in the form of early afterdepolarizations and ultimately torsades de pointes.¹⁶ QT prolongation occurs with either reduced outward or increased inward currents, and it accounts for the increased risk associated with K^+ channel-blocking antiarrhythmic drugs,^{17,18} as well as the congenital long-QT syndrome.¹⁹ Reports indicate that the incidence of drug-induced long-QT syndrome with azithromycin is less than that seen with the older macrolides and that torsades de pointes is unusual. Third, patients can develop polymorphic ventricular tachyarrhythmias in the setting of a normal QT interval,^{13,20,21} exemplified by bidirectional ventricular tachycardia (VT) that can occur with digitalis therapy. Digoxin-mediated inhibition of the $\text{Na}^+–\text{K}^+$ ATPase causes intracellular Na^+ loading, disrupting intracellular Ca^{++} homeostasis via reverse mode $\text{Na}^+–\text{Ca}^{++}$ exchange, with increased intracellular Ca^{++} causing delayed afterdepolarizations and VT. Similarly, spontaneous Ca^{++} release from the sarcoplasmic reticulum during catecholaminergic polymorphic VT results in polymorphic VT in the absence of QT prolongation.

A previous case report suggested that azithromycin could cause a distinct, previously undescribed type of drug-induced proarrhythmia, manifested by rapid polymorphic VT in the absence of ECG or structural abnormalities: specifically the QT was normal, with no pause before the tachycardia.⁶ We describe a similar case and together, these data imply a novel

proarrhythmic mechanism. Accordingly, we examined the electrophysiological effects of azithromycin *in vivo* and *in vitro* to investigate the molecular basis for this unusual form of drug-mediated proarrhythmia.

Methods

Reagents

Azithromycin was provided by Pfizer, Inc (Groton, CT) and dissolved in dimethyl sulfoxide to generate a 100 mmol/L stock solution (stored at -20°C). The stock solution was serially diluted in bath solution to the final concentrations before each experiment. The drug was prepared for oral administration as described previously.²²

Cell Preparations

The effects of azithromycin on the ionic currents under study were investigated using heterologously expressed human channels and cardiomyocytes. For cardiomyocyte studies, the species selected for experimentation was one that would optimize recording conditions for the specific current under study.

Human embryonic kidney (HEK 293) cells that stably expressed either human KCNH2 (hERG) or human SCN5A were kindly provided by Dr Craig January (University of Wisconsin) and Dr Alfred George (Northwestern University), respectively. A Chinese hamster ovary (CHO) cell line stably expressing KCNQ1 and KCNE1 to generate I_{Ks} currents was also provided by Dr George. The construct encoding the human Kir2.1 channel was kindly provided by Dr Antonin Lapoli, with transient transfection in HEK cells as reported previously.^{23,24} HEK293, CHO, and HL-1 cells were cultured as described.^{23,25–28}

Isolation of rabbit ventricular myocytes was performed using the method of Bassani²⁹ with minor modifications. Murine left ventricular myocytes were prepared from 10- to 12-week-old male mice as previously described.³⁰ The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

Data Acquisition

Mouse ECGs

A DSI (Data Science International, St Paul, MN) telemetry system was used to monitor and collect ECG data from conscious, freely moving laboratory mice. C57BL/6 mice (age 10–12 weeks) were anesthetized using ketamine 100 $\mu\text{g}/\text{g}$ and xylazine 10 $\mu\text{g}/\text{g}$ injected IP to place a radio transmitter (EA-F20) in the abdominal cavity. The mouse ECG telemetry system contains 2 electrical ECG leads connected to a radio transmitter with subcutaneous electrodes in a lead I configuration. On activation of the transmitter by a magnet, the electrical signals were transmitted wirelessly to a nearby receiver (RPC-1) attached to an amplifier (MX2) and computer system for data acquisition (Ponemah v6.10; sampling frequency 2 KHz), storage, and analysis. Animals were allowed to recover for at least 5 days after surgery before experimentation. Each mouse served as its own control with resting ECG recorded for at least 15 to 30 minutes before any intervention. For IP administration, azithromycin 50 mg/kg was injected, followed by 100 mg/kg IP 1 hour later. ECG monitoring continued for at least 1 hour after the second injection. A separate group of mice were treated with oral azithromycin for 3 days, using a dose that was efficacious in treating infections.³¹ Baseline ECG was recorded, azithromycin 50 mg/kg was administered by oral gavage, and the ECG was recorded for 2 hours. This was repeated for 2 or 3 additional days.

Action Potentials

Spontaneous action potentials were recorded at 37°C from HL-1 cells as previously described (Data Supplement).²⁸ Cells chosen for experimentation had a resting membrane potential of at least -55 mV , overshoot exceeding 20 mV, regular rhythmicity, and a stable spontaneous cycle length for at least 5 minutes.

Ionic Currents

All currents were recorded at room temperature ($22\pm1^\circ\text{C}$) in the whole-cell configuration with an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA). Currents were low-pass filtered at 5 kHz and digitized with a DigiData 1320A. Capacitance and 80–95% series resistance were routinely compensated. Leak subtraction was completed using user-specified after-the-fact leakage correction of pCLAMP. Na^+ current, rapidly activating (I_{Kr}) and slowly activating (I_{Ks}) delayed rectifier K^+ currents, L- and T-type Ca^{2+} currents, and inward rectifier current (I_{K1}) were recorded as detailed in the [Data Supplement](#). Pipettes were pulled (Sutter Instrument, Novato, CA) using Borosilicate glass having tip resistances of 0.5 to 1 $\text{M}\Omega$.

Pulse Protocol and Solutions

These are described in the [Data Supplement](#) and illustrated in the representative figures.

Data Analysis

ECG Data

Mouse ECG waveforms over a 10-second period were averaged to obtain measurements of the RR, PR, QRS, QT, and QTc intervals and heart rate using LabChart (version8.1.5; AD Instruments, Colorado Springs, CO) and are presented as mean \pm SEM. QTc was calculated using the normalized Bazett formula ($\text{QTc}=\text{QT}/[\text{RR}/100]^{1/2}$).³²

Action Potentials and Ionic Currents

Data were analyzed using Clampfit 10.4 software (Molecular Devices), Excel 2010 (Microsoft, Redmond, WA), and Origin 9.0 (OriginLab Corporation, Northampton, MA), with action potential measurements obtained as previously described ([Data Supplement](#)).⁷ Current amplitudes were normalized by cell capacitance and expressed as current density. All activation and inactivation curves were fitted with a single Boltzmann function as follows: $y=A_2+(A_1-A_2)/(1+\exp((V_m-V_{1/2})/k))$, where A_1 is maximum current, A_2 is the minimum current, $V_{1/2}$ is the half-activation or half-inactivation potential (ie, half availability), V_m is test voltage, and k is slope factor. Fractional blockade was defined as follows: $f=1-I_{\text{drug}}/I_{\text{con}}$, where I_{con} and I_{drug} are current amplitudes in the absence and presence of azithromycin, respectively. Dose-response curves were fit by the equation: $y=B_1+(B_2-B_1)/(1+10^{(\log x_0-x)/p})$, where B_1 is the bottom asymptote, B_2 is the top asymptote, $\log x_0$ is IC_{50} , x is azithromycin concentration, and p is hill slope.

Whole-Exome Sequencing

After informed consent, DNA was extracted from peripheral blood leukocytes and protein-coding regions targeted using the Agilent SureSelect All Exon V3 capture kit. High-throughput DNA sequencing was then performed using the Illumina HiSeq 2500 instrument with the paired-end sequencing protocol generating just under 100 million short (75 mb) sequence read pairs.³³ Raw sequence reads were aligned to the human reference (hg19) using the Burrows-Wheeler Aligner (0.6.9) aln algorithm after hard trimming to 50 bp. Duplicates were flagged using the Picard suite of tools. Postprocessing of the aligned data was done using the (GATK v1.6–19) including local realignment and base quality recalibration. Variant detection was done using the UnifiedGenotyper from GATK and called collectively to a multisample variant call format file. Low-quality sites were flagged using the VariantFiltrationWalker in agreement with the GATK BestPractices using filter thresholds Qual <50, QD <5, and AB >0.75.

mRNA Quantification

Real-time quantitative reverse transcription polymerase chain reaction was performed in HEK cells expressing SCN5A after 24-hour exposure to azithromycin or vehicle as described previously ([Data Supplement](#)).^{34,35}

Protein Quantification

Western analysis to quantify SCN5A protein in HEK cells was performed as described ([Data Supplement](#)).^{35–37} Both whole-cell lysate and biotinylated fraction were analyzed to quantify total cellular and plasma membrane-bound protein, respectively.

Azithromycin Plasma Concentrations

At 15 minutes after IP injection (when electrophysiological effects were maximal), animals were anesthetized with isofluorane, and blood was harvested by cardiac puncture. During oral administration, animals were euthanized at 90 minutes (the time to maximal plasma concentration;³¹ n=4) or at 24 hours (at trough or predose; n=6) after the last dose for measurement of azithromycin plasma concentration. Plasma was isolated for extraction and analysis of azithromycin concentration using liquid chromatography–tandem mass spectrometry as described.³⁸

Statistical Analysis

Data represented using a continuous variable were summarized using mean and SE for each group. For group comparisons of a repeated

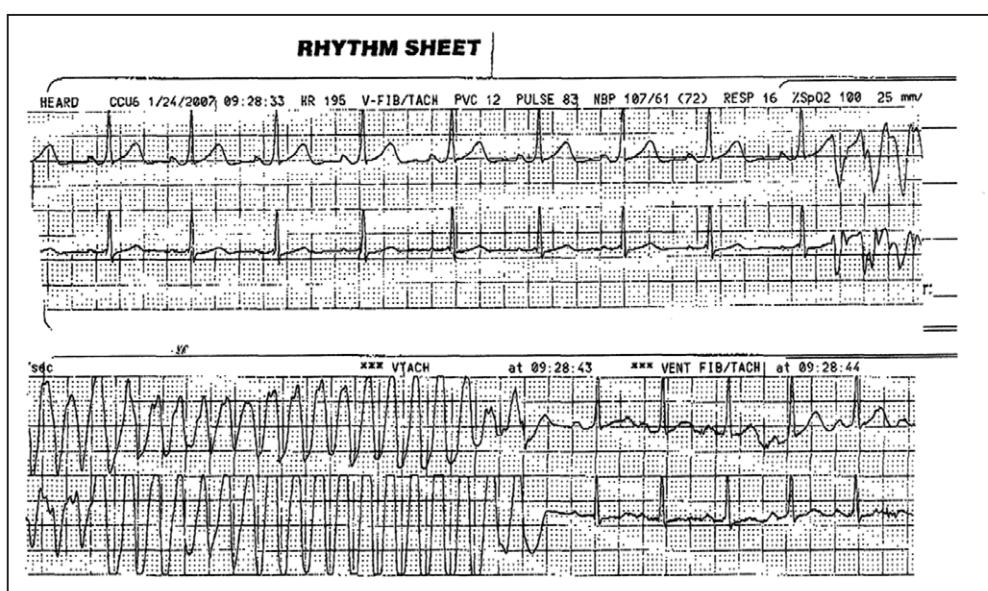


Figure 1. Azithromycin-induced polymorphic ventricular tachycardia (VT) in a 24-y-old woman with no structural heart disease and a normal ECG. The arrhythmias resolved with stopping the drug.

measure, the nonparametric Wilcoxon signed-rank test was applied. For independent group comparisons, the nonparametric Wilcoxon rank-sum test was used. Exact *P* values were calculated for these tests. For comparisons between multiple dose groups or time groups, *P* values were adjusted using the Bonferroni correction method. For each parameter of mouse ECG data, a linear regression with robust SEs and consideration of mouse cluster analysis were also performed. The mean differences between dose groups and the confidence intervals were estimated. Two-sided nominal level of 0.05 was considered as statistically significant. Analyses were performed using R 3.2.4 (<https://www.R-project.org/>), Origin Pro 2016 (Origin Laboratory Corporation, Northampton MA 01060), and Microsoft Excel 2010.

Results

Case Report

The patient was a 24-year-old black female with no medical history who was treated for 5 days with azithromycin (Zithromycin Z-Pak) for an upper respiratory infection. She was taking no other medications or supplements. Within 24 hours after discontinuing azithromycin, she experienced recurrent episodes of syncope and presented to a local emergency department. Her ECG on admission was normal (Figure I in the Data

Supplement). A rhythm strip obtained during a syncopal episode demonstrated rapid, polymorphic VT in the setting of a normal QT interval (Figure 1). She remained off azithromycin, and the VT resolved over 3 days. Cardiac magnetic resonance imaging was within normal limits. The patient received an implantable cardioverter-defibrillator before discharge. Whole-exome sequencing was performed, and there were no rare nonsynonymous variants identified in genes previously associated with inherited arrhythmia syndromes.

Acute Electrophysiological Effects of Azithromycin in Conscious Mice

As an initial step to investigate the electrophysiological effects of azithromycin *in vivo*, the drug was injected IP into conscious mice during continuous ECG monitoring. Within 15 minutes, azithromycin produced diffuse, dose-dependent depressant effects on cardiac automaticity and conduction (Figure 2; Table 1), with reversible slowing of heart rate (Figure II in the Data Supplement), and significant increases in the PR, QRS, QT, and QTc intervals (Table I in the Data Supplement). Similar findings were observed after oral administration, with sinus slowing and an increase in all ECG intervals

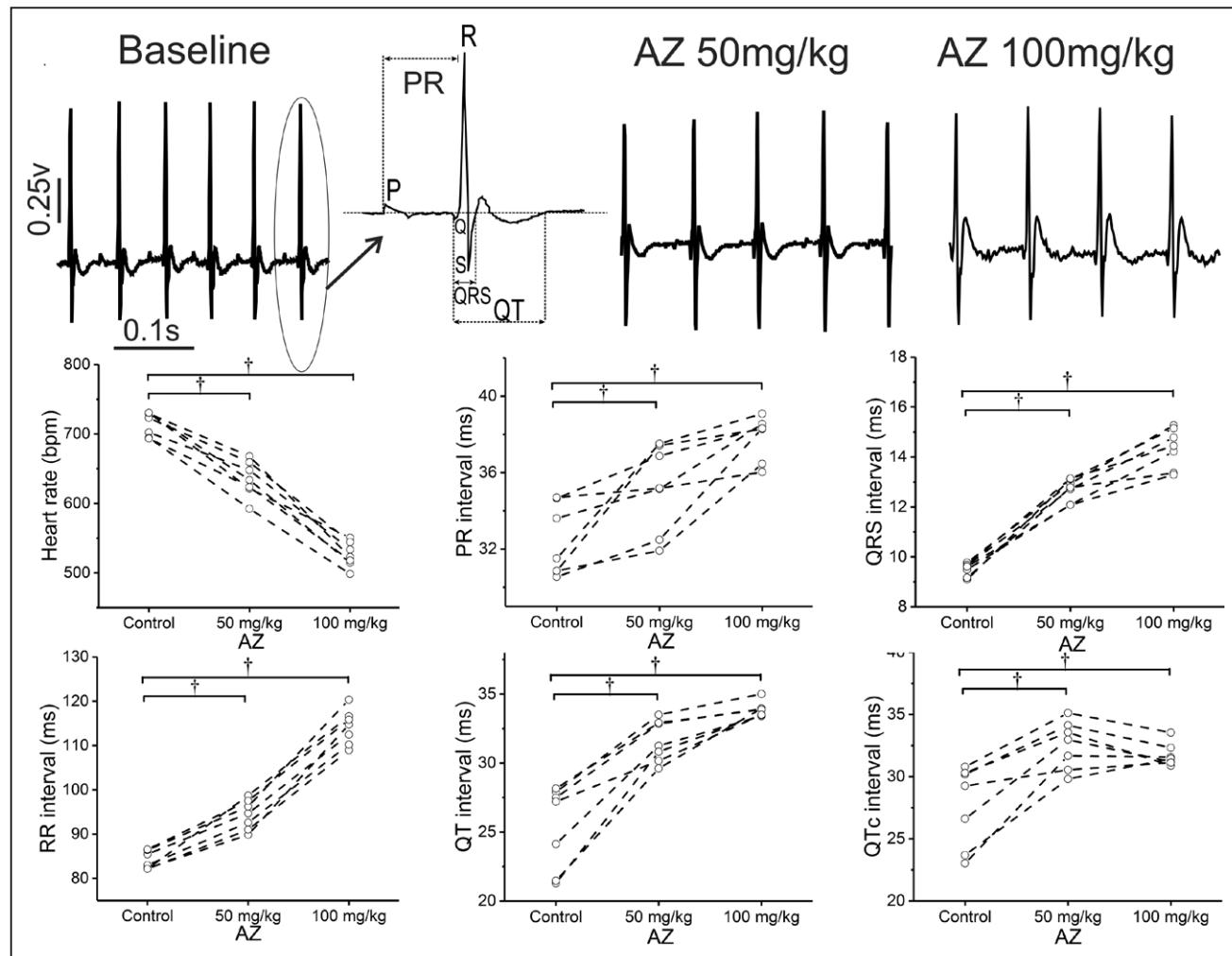


Figure 2. Effects of IP azithromycin (AZ) on the ECG of conscious adult mice. Mice received IP injection of AZ (50 mg/kg, followed in 60 min by 100 mg/kg). Representative ECG tracings (and interval measurements) are shown in the top, with parameter data for the group shown in the bottom ($n=7$). With both doses of AZ, heart rate declined, along with an increase in the PR, QRS, and QT/QTc intervals. $\dagger P < 0.01$.

Table 1. Summary Data of Azithromycin Effects on the ECG of Conscious Mice

	Heart Rate, beats per min	PR, ms	QRS, ms	QT, ms	QTc, ms
Control	715±7	32.4±0.7	9.5±0.1	25.4±1.2	27.7±1.2
AZ 50 mg/kg	637±8†	35.2±0.9*	12.7±0.2†	31.6±0.6†	32.5±0.7†
AZ 100 mg/kg	526±7†	37.9±0.4†	14.4±0.3†	33.9±0.2†	31.7±0.4*

n=7 mice. Wilcoxon signed-rank test, normalized Bazett: QTc=QT/(RR/100)^{1/2}. AZ indicates azithromycin.

*P<0.05.

†P<0.01.

within 60 minutes of dosing (Figure III and Table II in the *Data Supplement*) and to a lesser extent 24 hours after dosing. These results are consistent with the effects of azithromycin overdose in humans, and they imply that acute administration of azithromycin blocks multiple depolarizing and repolarizing ionic currents in cardiomyocytes.

In separate groups of mice, plasma azithromycin concentrations were determined after IP injection (at 15 minutes) and oral dosing (at 90 minutes when plasma concentrations were maximal,³¹ and at 24 hours). Values were 0.09±0.03 and 0.26±0.4 µg/mL after 50 and 100 mg/kg IP injection, respectively (n=3 each). For oral administration, plasma concentrations were 0.12±0.01 and 0.07±0.01 µg/mL at 90 minutes and 24 hours post-dosing (n=4 and 6, respectively). For comparison, peak plasma concentrations in humans are shown during chronic oral dosing with the 3 azithromycin regimens available commercially (Table III in the *Data Supplement*, from the product package insert). Thus, azithromycin concentrations resembling those seen clinically were achieved during these experiments. However, the depressant ECG effects seen in the mouse are not observed clinically with oral administration in humans, demonstrating substantial interspecies pharmacokinetic differences.

Acute Effects of Azithromycin on Cardiomyocyte Action Potentials

To further confirm these effects for cardiomyocytes, we examined the effects of azithromycin on HL-1 cells, a cultured cardiac cell line that displays spontaneous automaticity. Acute exposure caused slowing of beat rate, a reduction in the phase 0 upstroke slope, and prolonged repolarization (Table 2; Figure IVA through IVC in the *Data Supplement*), consistent with the diffuse depressant effects observed in vivo.

Reduction of SCN5A Currents With Acute Exposure

The QRS widening in vivo and reduced phase 0 upstroke in cardiomyocytes implied block of cardiac Na⁺ current by azithromycin. In HEK cells expressing human SCN5A channels, azithromycin caused rapid block of I_{Na}, with an IC₅₀ of ≈110 µmol/L, findings that were further confirmed in mouse ventricular myocytes (Figure 3A through 3D; Table 3). Suppression of Na⁺ current was accompanied by a depolarizing shift in the voltage dependence of channel activation and a hyperpolarizing shift in inactivation (Figure 3E and 3F; Table 3), changes that can account at least to some extent for the reduction in I_{Na} observed.³⁹

Paradoxical Time-Dependent Increase in Peak I_{Na}

Recent studies have highlighted the importance of time-dependent effects of electrophysiologically active agents on cardiac I_{Na}.^{40,41} To determine whether such properties might also be present for azithromycin, we characterized Na⁺ current in cells chronically exposed to the drug (for 24 hours), compared with control (vehicle-exposed) cells. Interestingly, under these conditions, peak SCN5A current density was increased at all test potentials examined, with upregulation of ≈2-fold at -20 mV (Figure 4A and 4B; Table 4). As for acute exposure, the voltage dependence of channel gating was also modulated (Figure 4C and 4D; Table 4). However, in this case, these effects could not contribute to potentiation of cardiac I_{Na}. These findings were replicated in HL-1 cardiomyocytes, with a substantial increase in

Table 2. Effects of AZ on Action Potentials

		CL, ms	Peak, mV	MDP, mV	DDT, ms	Phase 0 Slope, mV/ms	APD ₁₀₀ , ms	APD ₉₀ , ms	Beat Rate, beats per min
Acute (n=14)									
Control	Mean SE	308.3 2.9	107.7 1.5	-63.8 0.8	19.6 1.1	2.45 0.13	288.6 2.8	277.0 2.8	194.9 1.8
AZ 100 µmol/L	Mean SE	353.7† 3.7	89.2† 1.8	-59.1† 1.0	39.6* 2.4	1.18* 0.05	314.1† 4.1	299.9† 4.1	169.9† 1.8
Chronic (n=8)									
Control	Mean SE	291.1 8.9	101.0 2.1	-63.2 0.5	18.8 0.8	2.5 0.1	272.3 8.3	256.4 8.1	207.5 11.1
AZ 100 µmol/L for 24 h	Mean SE	216.7† 13.9	99.5 1.2	-60.6† 0.3	15.1* 1.2	4.0* 0.6	201.6† 12.9	185.5† 12.8	285.0† 13.4

Wilcoxon signed-rank test. APD₉₀ indicates action potential duration at 90% of repolarization; APD₁₀₀, action potential duration at 100% of repolarization; AZ, azithromycin; CL, cycle length; DDT, diastolic depolarization time; and MDP, maximum diastolic potential.

*P<0.05.

†P<0.01.

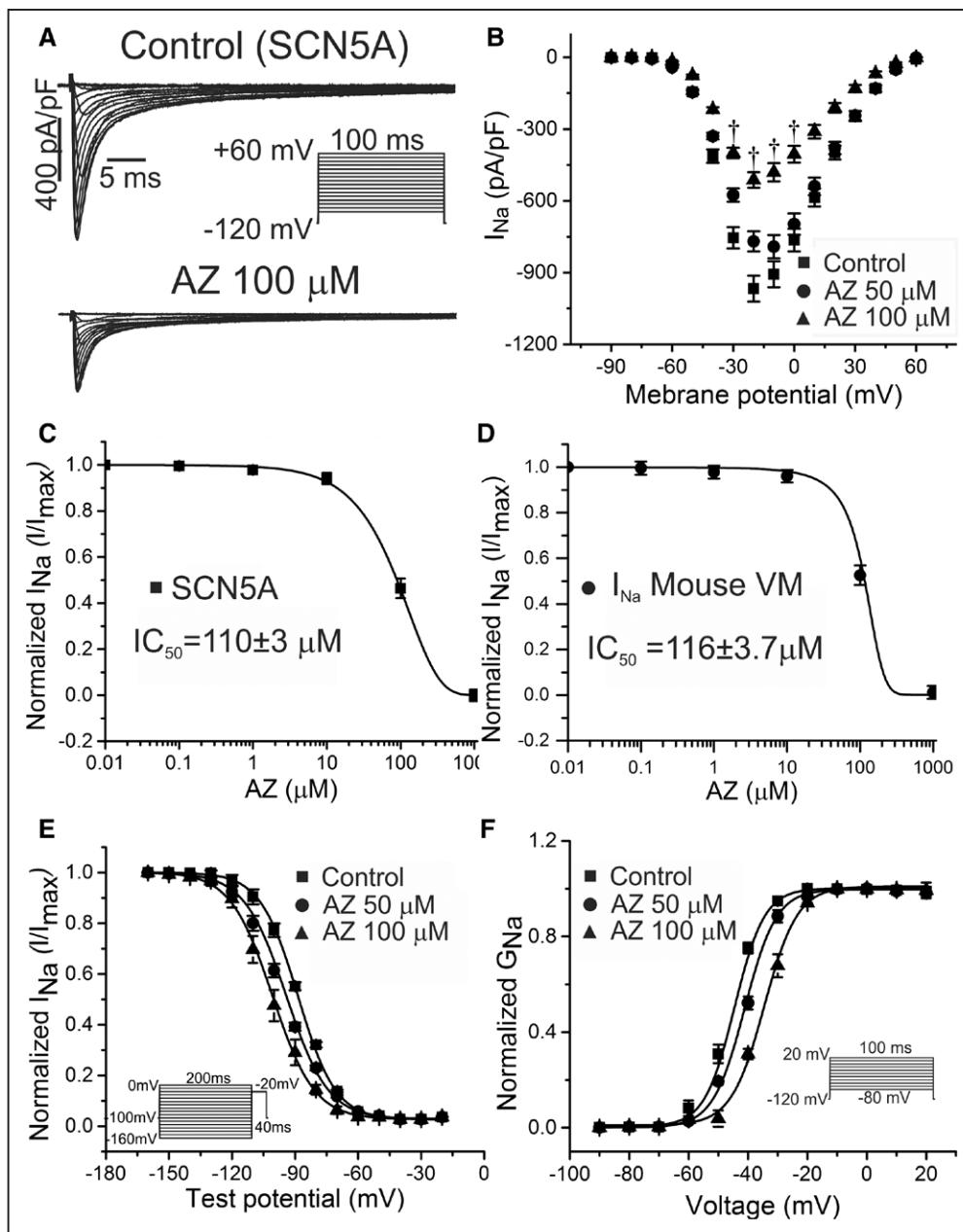


Figure 3. Concentration-dependent block of human and mouse cardiac Na⁺ currents with acute azithromycin (AZ) exposure. **A** and **B**, In human embryonic kidney (HEK) cells expressing human SCN5A, acute exposure to AZ significantly reduced I_{Na} (from -967 ± 54 to -769 ± 49 and -512 ± 38 pA/pF for AZ 50 and 100 μ mol/L, respectively, at -20 mV [n=18, 14]; $\dagger P < 0.01$; representative current tracings in **(A)** and current-voltage plot of summary data in **(B)**). **C** and **D**, The concentration-response curve demonstrated an IC_{50} of $110 \pm 3 \mu$ mol/L for SCN5A currents (**C**; n=6 for each concentration), with similar results obtained using mouse ventricular myocytes (**D**; VM; $IC_{50} = 116 \pm 3.7 \mu$ mol/L; n=5 for each concentration). **E** and **F**, Using the voltage-clamp protocols shown in the insets, normalized steady-state inactivation (**E**) and activation (**F**) curves for SCN5A were generated and fit with a Boltzmann function. AZ caused small but significant shifts in the hyperpolarizing and depolarizing directions, respectively (n=14–32; $\dagger P < 0.01$ for AZ 100 μ mol/L in **E** and **F**).

peak Na⁺ current and similar gating shifts on chronic azithromycin exposure (Table 4; Figure V in the [Data Supplement](#)). Consistent with this effect, the phase 0 upstroke slope was increased in HL-1 cardiomyocyte action potentials under similar conditions (Table 2; Figure IVD and IVE in the [Data Supplement](#)).

To explore the mechanism of azithromycin-mediated potentiation of Na⁺ current, experiments were performed using real-time quantitative reverse transcription polymerase chain reaction and Western analysis to determine whether the drug altered the production of SCN5A mRNA, total cellular

channel protein, or protein at the cell surface (using biotinylation). However, azithromycin had no effect on any of these properties for SCN5A (Figure VI in the [Data Supplement](#)).

Time-Dependent Increase in Late I_{Na}

Rarely, azithromycin causes drug-induced long-QT syndrome. One potential mechanism is an increase in the Na⁺ window current, defined by overlap of the activation and inactivation curves, or a window of potentials over which a fraction of Na⁺ channels are activated but not inactivated. However,

Table 3. Acute Effects of AZ on SCN5A Currents

	Peak I_{Na} (n=14–18)	Activation (n=14–32)		Inactivation (n=14–32)		Late I_{Na} (n=12–16)
	pA/pF	$V_{1/2}$ mV	k	$V_{1/2}$ mV	k	pA/pF
CTL	-967±54	-44.6±1	5.0±0.5	-88.6±0.6	7.9±0.1	0.17±0.02
AZ 50 μ mol/L	-769±49	-41.1±1	5.3±0.3	-94.2±0.6	9.9±0.2	0.23±0.02
AZ 100 μ mol/L	-512±38†	-34.5±1†	5.6±0.3	-100.8±0.6†	9.9±0.3	...

Wilcoxon signed-rank test. AZ indicates azithromycin; CTL, control; and k, slope factor.
† $P<0.01$.

azithromycin caused either no change or a reduction, rather than an increase, in the window current with acute and chronic exposure (Figure VII in the Data Supplement).

Because an increase in late Na^+ current can also prolong repolarization, we investigated the effects of azithromycin on this parameter using TTX subtraction in HEK cells expressing SCN5A (Figure 5A). Acute exposure to azithromycin had minimal effect on amplitude of late I_{Na} (Figure 5B; Table 3); however, chronic exposure for 24 hours caused doubling of the late Na^+ current (Figure 5A and 5B; Table 4). Potentiation of late I_{Na} was greater than the increase in peak I_{Na} , illustrated by an increase in the ratio of these parameters (Figure VIII in the Data Supplement). The effects of chronic azithromycin exposure to increase both peak and late I_{Na} occurred at lower concentrations ($IC_{50}=40.9$ and 62.2 $\mu\text{mol/L}$, respectively; Figure 5C and 5D) than those causing acute I_{Na} block

($IC_{50}=110–116 \mu\text{mol/L}$; Figure 3) implying greater clinical relevance during therapeutic dosing.

Minimal Effects on the Rapidly and Slowly Activating Delayed Rectifier K^+ Currents

Most drugs prolong cardiac repolarization by blocking the rapidly-activating delayed rectifier K^+ current, I_{Kr} . In HEK cells expressing hERG, K^+ currents displayed only mild block by azithromycin, with an IC_{50} of 219±21 $\mu\text{mol/L}$ (Figure 6; Table 5). Similarly, there was minimal effect of the drug on the slowly activating delayed rectifier, I_{Ks} (Figure 7; Table 5; $IC_{50}=184±12 \mu\text{mol/L}$). Given that these concentrations are 3- to 4-fold higher than that for potentiation of late I_{Na} , azithromycin-mediated QT prolongation most likely results from increased late Na^+ current, rather than K^+ channel block.

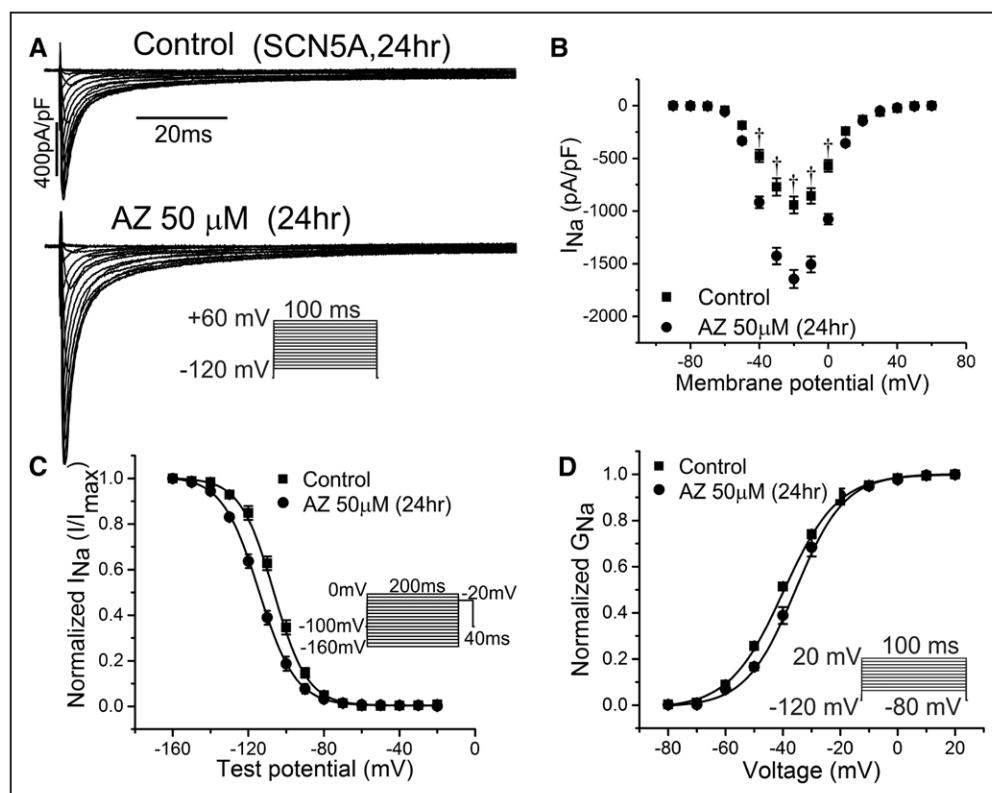


Figure 4. Increased peak Na^+ current with chronic azithromycin (AZ) exposure. **A** and **B**, For SCN5A currents in human embryonic kidney (HEK) cells, incubation with AZ (50 $\mu\text{mol/L}$) for 24 hours significantly increased I_{Na} (from -942±80 pA/pF to -1644±76 pA/pF at -20 mV; n=13 each; † $P<0.01$). **C** and **D**, In the same preparation, similar shifts were observed in the steady-state inactivation and activation curves, as those seen with acute exposure (for inactivation, -106.6±1.2 mV to -119.9±1.2 mV for control and AZ 50 $\mu\text{mol/L}$, respectively; for activation, -41.0±2 to -35.3±1 mV; n=14 and * $P<0.05$ for both).

Table 4. Chronic (24 h) Exposure of SCN5A Currents to AZ

(n=8–14)	Peak I_{Na}	Activation		Inactivation		Late I_{Na}
	pA/pF	$V_{1/2}$ mV	k	$V_{1/2}$ mV	k	pA/pF
CTL (SCN5A)	-942±80	-41.0±2	9.7±1	-106.6±1.2	9.1±0.4	0.18±0.02
AZ 50 $\mu\text{mol/L}$	-1644±76†	-35.3±1*	8.4±1	-119.9±1.2*	9.5±0.4	0.48±0.03†
IC_{50} $\mu\text{mol/L}$	53.3±1.6	62.2±1.5
CTL (HL-1 cells)	-81±19	-49.8±1	8.1±1	-93.8±1.3	9.5±0.4	0.21±0.02
AZ 50 $\mu\text{mol/L}$	-135±22†	-47.6±1*	7.9±1	-98.6±1.4*	10.6±0.4	0.49±0.02†

Wilcoxon rank-sum test. AZ indicates azithromycin; CTL, control; and k, slope factor.

* $P<0.05$.

† $P<0.01$.

Block of Cardiac Calcium Currents and I_{K1}

Because azithromycin slows sinus node automaticity and prolongs atrioventricular nodal conduction in vivo, we tested the hypothesis that the drug blocks cardiac Ca^{++} currents. Using rabbit ventricular myocytes, nifedipine-sensitive L-type Ca^{++} currents were suppressed by azithromycin ($IC_{50}=67±4$ $\mu\text{mol/L}$; Figure 8A and 8B; Table 5), while mibepradil-sensitive T-type Ca^{++} currents were minimally affected (IC_{50} not determined; Figure 8C and 8D; Table 5).

Azithromycin increased the maximal diastolic potential in HL-1 cells during both acute and chronic exposure (Table 2), implying an effect on the inward rectifier current I_{K1} . Indeed, for HEK cells expressing Kir2.1 and for HL-1

cells, azithromycin suppressed Kir and I_{K1} currents, respectively ($IC_{50}=43.8$ $\mu\text{mol/L}$ for Kir2.1 currents; Table 5; Figure IX in the Data Supplement).

Discussion

Azithromycin is a widely prescribed macrolide antibiotic that has been linked to an increased risk of sudden cardiac death and cardiovascular death.¹¹ In contrast to other macrolides, torsades de pointes has been rarely reported for azithromycin.^{3–5,7–9} Thus, the cause of these adverse events has remained poorly defined, prompting the present investigation. As demonstrated by the case presented here, azithromycin can cause a unique, drug-mediated proarrhythmic syndrome characterized

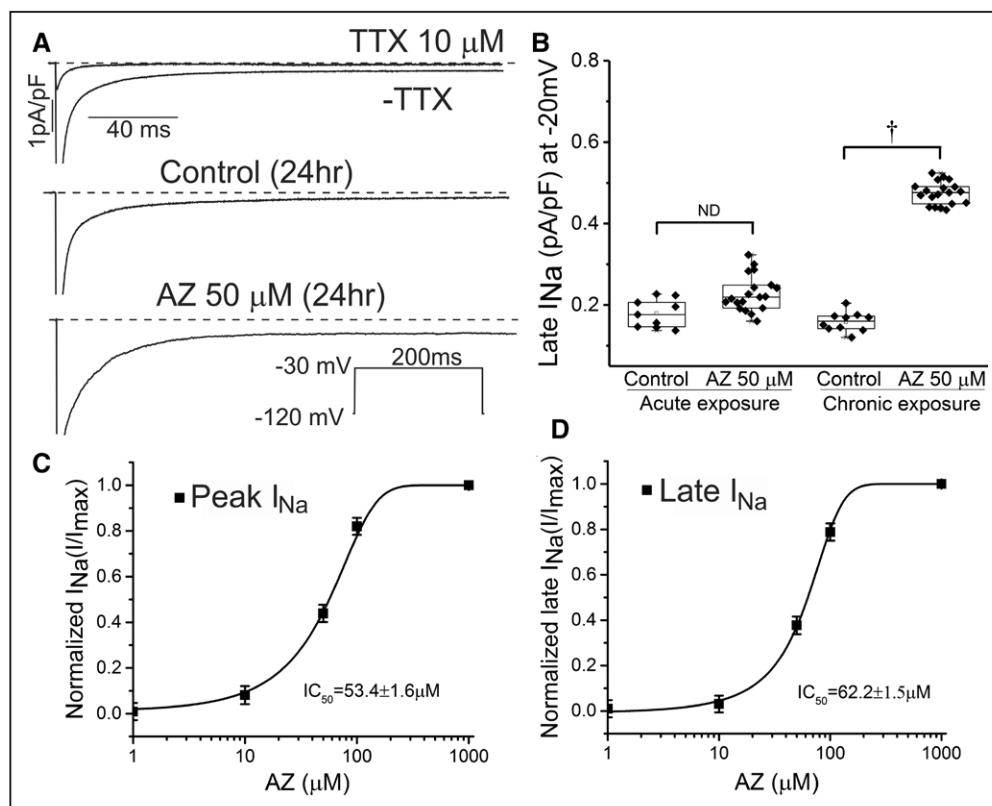


Figure 5. Time- and concentration-dependent increases in late and peak I_{Na} . **A**, For SCN5A currents, examples of tetrodotoxin-sensitive Na^{+} current (**top**) are shown in the absence (control; **middle**) and presence of 24-h incubation with azithromycin (AZ; 50 $\mu\text{mol/L}$; **bottom**). **B**, Summary data demonstrate a significant increase in late I_{Na} (from $0.18±0.02$ to $0.48±0.03$ pA/pF; n=6; † $P<0.01$) with chronic (24 h) but not acute exposure. **C** and **D**, The concentration–response relationships for the increase in peak (**C**) and late (**D**) SCN5A currents after a 24-h exposure to azithromycin are illustrated.

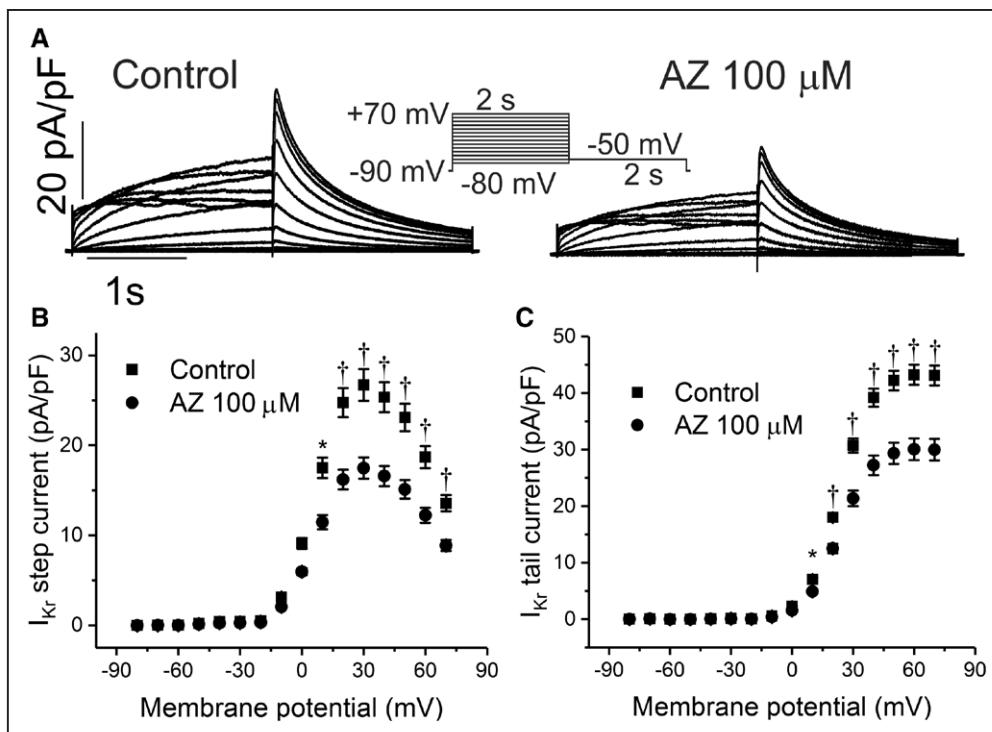


Figure 6. Effects of azithromycin (AZ) on hERG currents (I_{K_r}). **A**, In human embryonic kidney (HEK) cells expressing human KCNH2 (hERG), K⁺ currents were recorded in the absence and presence of AZ 100 μ M/L. **B** and **C**, Current–voltage relationships are shown for steady-state (**B**) and tail (**C**) current before and after acute exposure to AZ, with a reduction of 34% and 30% at +60 mV, respectively ($n=15$; IC₅₀ 219±21 μ M/L; * $P<0.05$, † $P<0.01$).

by rapid, polymorphic VT in the absence of QT prolongation or demonstrable cardiac structural abnormalities.⁶ We found that chronic drug exposure markedly potentiates cardiac Na⁺ current, which would increase intracellular [Na⁺] and subsequently promote dysregulation of intracellular [Ca⁺⁺], providing a unifying mechanism for this unusual syndrome that likely contributes to azithromycin-mediated sudden cardiac death.

It is well recognized that conditions associated with intracellular Na⁺ loading, most notably digoxin therapy and ischemia, alter intracellular Ca⁺⁺ homeostasis via the Na⁺-Ca⁺⁺ exchanger activity, with removal of Na⁺ in exchange for Ca⁺⁺, leading to delayed afterdepolarizations and triggered arrhythmias.³² Abnormal intracellular Ca⁺⁺ homeostasis due to sarcoplasmic reticulum Ca⁺⁺ leak is the hallmark of catecholaminergic polymorphic VT, the clinical syndrome that

most closely resembles this type of azithromycin proarrhythmia. Recent evidence further supporting the arrhythmogenic effects of cellular Na⁺ loading derives from a transgenic mouse model in which peak Na⁺ current was increased.⁴² Mutations in *Scn1b*, encoding the Na⁺ channel β_1 subunit, have been linked to inherited arrhythmia syndromes, including sudden infant death syndrome and unexplained death in epilepsy.^{43,44} Mice with cardiac-specific deletion of *Scn1b* demonstrated enhanced peak I_{Na} , delayed afterdepolarizations, and polymorphic VT without APD prolongation, which was responsive to treatment with tetrodotoxin. Moreover, catecholaminergic polymorphic VT was recently linked to a gain-of-function mutation in *Scn5a* that increased Na⁺ influx by augmenting the Na⁺ channel window current.⁴⁵ Along with other studies,^{46,47} these data provide compelling evidence that a generalized increase in cardiac Na⁺

Table 5. Acute Effects of AZ on Multiple Ionic Currents

n	I_{Na} (pA/pF)	I_{K_r} (pA/pF)		I_{K_s} (pA/pF)		I_{Ca} (pA/pF)	I_{Ca} (pA/pF)	I_{K_1} (pA/pF)
	14	15	SS	Tail	SS	Tail	Peak	Peak
Control	-967±54	26.8±3	43.0±4	316.7±42	49.6±3	-31±6	-85.1±19	-79.5±6
AZ 100 μ mol/L	-512±38†	17.6±2†	30.1±4†	221.6±22*	34.6±2*	-11±2†	-60.6±16*	-27.2±6†
% inhibition	47	34	30	30	30	65	29	66
IC ₅₀ , μ mol/L	110±3	219±21		184±12		66.5±4	...	43.8±3.2

Wilcoxon signed-rank test. AZ indicates azithromycin; Peak, peak current; SS, steady-state current; and Tail, tail current.

* $P<0.05$.

† $P<0.01$.

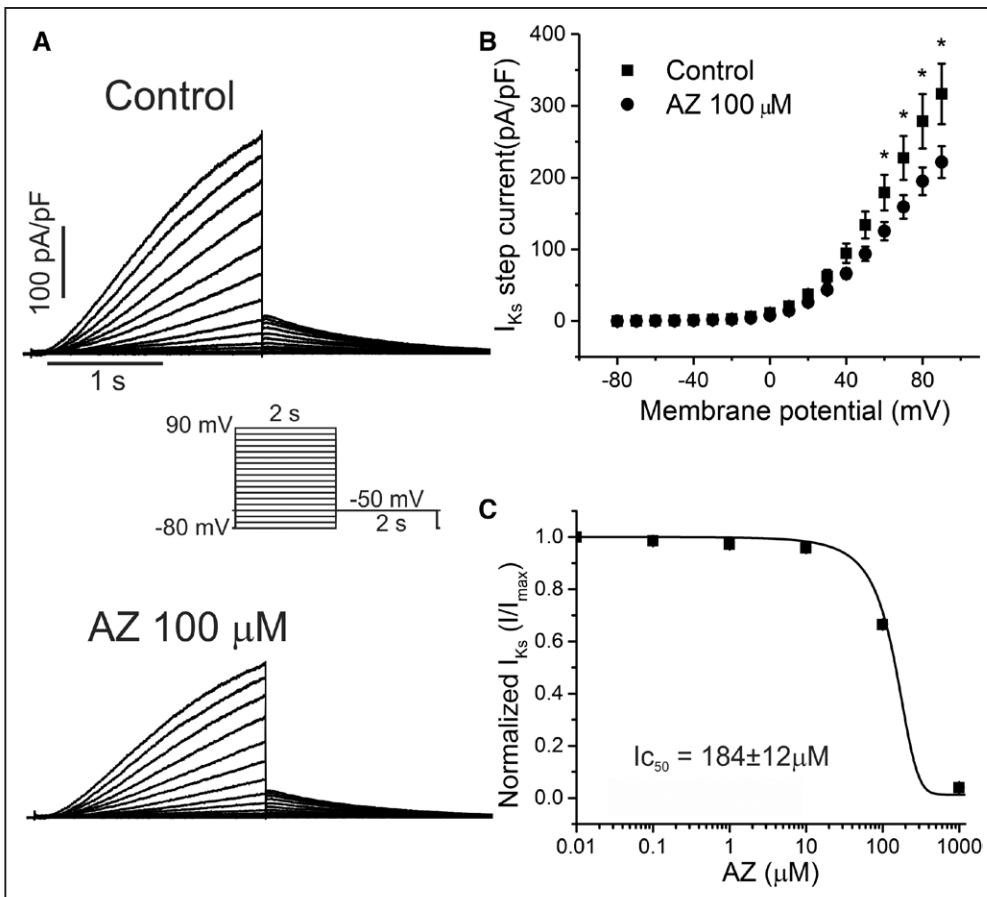


Figure 7. Minimal reduction in I_{Ks} current. **A**, In Chinese hamster ovary (CHO) cells stably expressing KCNQ1 and KCNE1, K^+ currents were recorded using the voltage-clamp protocol shown in the inset before and after acute exposure to azithromycin (AZ; 100 $\mu\text{mol/L}$). I_{Ks} steady-state current was suppressed by 30% at +80 mV ($n=12$; * $P<0.05$). **B**, Current–voltage plot of tail current demonstrated a 30% reduction at +80 mV ($n=12$; * $P<0.05$). **C**, The concentration–response curve demonstrated an IC_{50} of $184 \pm 12 \mu\text{mol/L}$.

current is arrhythmogenic by dysregulating intracellular Ca^{++} , with clinical features exemplified by catecholaminergic polymorphic VT that mimic the unusual form of drug-mediated proarrhythmia described here.

Azithromycin can cause life-threatening arrhythmias by at least 2 other mechanisms, although these seem to be rare. First, cases of drug-induced long-QT syndrome have been reported, but as noted above, documented torsades is unusual. Based on our results, potentiation of late I_{Na} by azithromycin appears to be responsible for this adverse effect, occurring at much lower concentrations than those required for K^+ channel block. Drug-induced long-QT syndrome is less common with azithromycin than with other drugs that also increase late I_{Na} , such as dofetilide.⁴¹ This is likely because many of the compounds that increase late Na^+ current also block I_{Kr} as well, and this is minimal with azithromycin. We speculate that concomitant block of cardiac Ca^{++} current by azithromycin might also provide an additional potential protective mechanism to prevent early afterdepolarizations and torsades.

Second, acute intravenous exposure¹⁴ (in one case causing overdose¹⁵) can cause wide-spread depression of cardiac conduction, resulting in marked sinus bradycardia, slowing of AV nodal and infranodal conduction with heart block, and prolonged ventricular repolarization. For conscious mice and cardiomyocytes, we found that acute exposure to azithromycin

had similar effects, with in vitro evidence of multi-ion channel block, providing a mechanistic basis for the electrophysiological effects of acute toxicity. Although these toxic effects have not been observed during oral dosing of azithromycin, we cannot rule out the participation of multi-ion channel block to drug-mediated sudden cardiac death. This is particularly true for acute Na^+ channel block, as well as block of I_{Kr} , which would elevate the myocyte resting potential leading to both enhanced automaticity and additional Na^+ channel inactivation.

An important consideration is the range of azithromycin concentrations that we studied. Several oral preparations of azithromycin have been marketed, and peak plasma concentrations during oral dosing range from ≈ 0.4 to $1.1 \mu\text{mol/L}$ (Table III in the [Data Supplement](#)).⁴⁸ However, plasma concentrations are misleading, as the drug accumulates within cells, achieving concentrations approaching $900 \mu\text{mol/L}$ in leukocytes and pulmonary tissue (Table III in the [Data Supplement](#)).⁴⁸ A previous study by the pharmaceutical sponsor reported similar accumulation of the drug in cardiac cells for mice receiving oral azithromycin ($200 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ for 10 days), with ≈ 30 - to 220-fold increase in concentration compared with plasma.²² On the basis of these data and known azithromycin plasma concentrations in patients, we investigated azithromycin concentrations of 0.1 to $1000 \mu\text{mol/L}$, to account for intracellular accumulation. Interestingly, amitriptyline also accumulates in

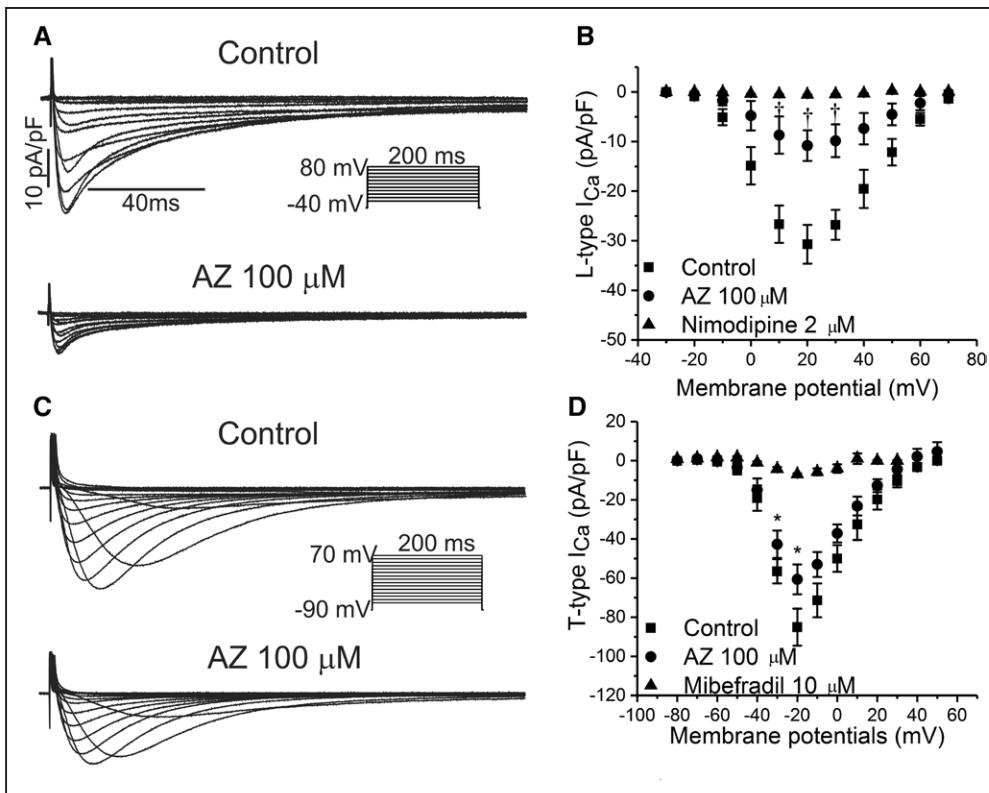


Figure 8. Effects of azithromycin (AZ) on Ca^{++} currents in rabbit ventricular myocytes. **A** and **B**, Families of currents and peak current–voltage relationships are illustrated for nimodipine-sensitive Ca^{++} currents, indicative of $I_{\text{Ca-L}}$, before and after acute exposure to AZ 100 $\mu\text{mol/L}$, with a 65% reduction at +20 mV ($n=7$; $\dagger P<0.01$; $IC_{50} 67 \pm 4 \mu\text{mol/L}$). **C** and **D**, Similar results are shown for mibepradil-sensitive Ca^{++} currents representing $I_{\text{Ca-T}}$ with 29% suppression at -20 mV ($n=8$; $*P<0.05$).

cardiomyocytes.⁴⁹ Intracellular concentrations were ≈ 5 -fold higher than extracellular concentrations, explaining the drug's higher potency in cardiomyocytes compared with RYR2 channels incorporated into lipid bilayers.

The mechanism for azithromycin-mediated potentiation of cardiac Na^{+} current remains unclear, as we found no evidence for increased production of SCN5A mRNA or total cellular protein, and there was no evidence for enhanced trafficking of channel protein to the cell surface. The alterations in channel gating, as well as the more selective increase in late I_{Na} , also argue against a nonspecific or generalized effect to increase Na^{+} channels at the membrane. We postulate that the increase in I_{Na} is mediated by effects that alter post-translational modification and protein–protein interactions, which could be complex.⁵⁰

In conclusion, chronic exposure to azithromycin markedly potentiates cardiac Na^{+} current to promote intracellular Na^{+} loading, providing a unifying mechanistic basis for the distinct type of proarrhythmia seen with this drug. These findings imply that preclinical screening of new compounds should include examination of their pharmacological effects on cardiac I_{Na} amplitude.

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Disclosures

None.

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Azithromycin Causes a Novel Proarrhythmic Syndrome

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SUPPLEMENTAL MATERIAL

DETAILED METHODS

Data Acquisition

Mouse ECGs. Methods using a telemetry system to monitor and collect ECG data from conscious, freely moving laboratory mice have been described previously.¹

Action Potentials. Spontaneous action potentials were recorded at 37°C from HL-1 cells as previously described.² The pipettes were filled with an internal solution containing (in mM): 120 K-aspartate, 25 KCl, 1 MgCl₂, 5 HEPES, 10 EGTA, 2 Na₂phosphocreatine, 4 Na₂ATP, 2 NaGTP, pH 7.2 (adjusted with 1M KOH). The bath solution was Tyrode's with: 145 NaCl, 4 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 HEPES, pH 7.4 (adjusted with 1M NaOH).

Ionic currents. Na⁺ current, rapidly (I_{Kr}) and slowly-activating (I_{Ks}) delayed rectifier K⁺ currents, L- and T-type Ca²⁺ currents, and inward rectifier current (I_{K1}) were recorded as described previously.^{3, 4, 5, 6, 7}

Recording I_{Na}. To record Na⁺ currents, the holding potential was -120mV. The voltage dependence of channel activation was measured using 200ms test pulses from -90mV to +80mV in 10 mV increments. To assay the voltage-dependence of steady-state inactivation, the membrane potential was stepped from -160mV to 0mV in 10mV increments (200ms pulses) using a holding potential of -100mV, followed by 40ms test pulse to -20mV. Late I_{Na} was averaged in a 3ms window (195-198ms after the pulse) before the capacity transient at the end of a 200ms depolarizing pulse at -20 mV and was quantified by subtraction of the current remaining after application of 30μM tetrodotoxin from the total current. For all preparations, the pipette solution contained the following: 10 NaF, 110 CsF, 20 CsCl, 10 EGTA, and 10 HEPES, pH 7.2

(adjusted with 1M CsOH).

SCN5A currents in HEK 293 cells. The bath solution contained the following: 145 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, and HEPES, pH 7.4 (adjusted with 1M NaOH).

I_{Na} in mouse ventricular myocytes and HL-1 cells. The bath solution contained: 20 NaCl, 1 MgCl₂, 110 CsCl, 5TEA-Cl, 0.1 CaCl₂, 2µM nimodipine, 200µM NiCl, HEPES and 10 glucose, pH 7.4 (adjusted with 1M NaOH).

Recording I_{Kr} and I_{Ks}. To record K⁺ currents, the cycle time for pulse protocols was 15s. For I_{Kr}, the holding potential was -90mV, and test potentials (2sec) were stepped from -80mV to +70mV, with tail currents recorded upon repolarization to -50mV for 2sec. For I_{Ks}, the holding potential was -80mV, and test potentials (2sec) were stepped from -80mV to +90mV, with tail currents recorded upon repolarization to -50mV for 2sec. Patch electrodes were filled with: K-DL-aspartate 120; KCl 25; MgCl₂ 1; EGTA 10; Na₂ phosphocreatine 2; Na₂ATP 4; NaGTP 2 and HEPES 5, pH 7.2 (adjusted with 1M KOH). HEK 293 cells were superfused with Tyrode's solution containing: 145 NaCl, 4KCl, 1 MgCl₂, 1.8 CaCl₂ and 10 HEPES, pH 7.4 (adjusted with 1M NaOH).

Recording Ca⁺⁺ Currents. For recording I_{Ca,L}, a holding potential of -40mV was used, , and the cycle time for pulse protocols was 5sec. Voltage was sequentially stepped for 200ms from -30mV to +80mV in 10mV increments. To record I_{Ca,T}, a holding potential of -90mV cells was used, with 200ms voltage steps from -80mV to +70mV (10mV increments). The pipette solution consisted of: 120 CsCl, 10EGTA-CsOH, 10 tetraethylammonium chloride (TEA-Cl), 1 Mg₂ATP, 5 phosphocreatine, 1 NaGTP, and 10 HEPES, pH 7.2 (adjusted with 1M CsOH. The bath solution contained: 140 TEA-Cl, 2 MgCl₂, 5CaCl₂, 10 HEPES, 10 glucose, pH 7.4 (adjusted with 1M CsOH).

Recording I_{K1} . For recording I_{K1} , a holding potential of -30mV was used, and the cycle time for pulse protocols was 5sec. Voltage was sequentially stepped for 150ms from -130mV to 0mV in 10mV increments. In HL-1 cells, I_{K1} was isolated by subtraction of the current remaining after application of 500 μ M BaCl₂ from the total current. In HEK cells expressing Kir2.1, or HL-1 cells, the pipette solution consisted of: 140 KCl, 5 K₂ATP, 1 EGTA, and 10 HEPES, pH 7.2 (adjusted with 1M KOH). The bath solution was Tyrode's with: 137 NaCl, 5.4 KCl, 0.5 MgCl₂, 0.3 CaCl₂, 0.16 NaH₂PO₄, 3 NaHCO₃, 5 HEPES, 5 glucose, pH 7.4 (adjusted with 1M NaOH).

mRNA Quantification

Total RNA was isolated from HEK cells expressing SCN5A after 24-hour exposure to azithromycin or vehicle using the RNAeasy Mini Kit with DNase treatment (Qiagen). cDNA was synthesized from 2 μ g of the RNA by use of the SuperScriptIII First Strand cDNA Synthesis System for RT-PCR with random hexamer primers (Life Technologies) and used as a template. To generate a standard curve for absolute quantification, *SCN5A* cDNA was PCR amplified and subcloned into the pGEM-T vector (Clontech, Mountain View, CA). cDNA and 5 different dilutions of the SCN5A vector were prepared with predesigned 6-carboxyfluorescein-labeled fluorogenic TaqMan probe and primers (Applied Biosystems) for *SCN5A* (Hs00165693.m1) or RPL19 (Hs02338565.qh) in triplicate in the same 94-well plate for real-time polymerase chain reaction amplification. Real-time quantitative RT-PCR was conducted with a 7900HT Real-Time Instrument (Applied Biosystems). Data were collected with instrument spectral compensation and analyzed by use of absolute quantification and a standard curve with SDS 2.4 software (Applied Biosystems). Each value was normalized to that for RPL19.

Western Analysis

Whole-cell lysates were prepared from HEK cells expressing SCN5A following 24-hour exposure to azithromycin or vehicle. Lysates were centrifuged at 10,000g for 5min, and protein content was analyzed with a bicinchoninic acid assay (Pierce Biochemicals, Rockford, IL). Proteins (20 µg) were separated by running the sample on a NuPage 4-12% Bis-Tris gel (Life Technologies, Carlsbad, CA). The protein was transferred to PVDF membranes (GE Healthcare Life Sciences, 0.45 um pore), blocked for 1hr with 10% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST) buffer at room temperature and then incubated overnight with antibodies targeting SCN5A (anti-pan NaV rabbit polyclonal antibody, 1:250; Alomone Labs, Israel), GAPDH (mouse monoclonal antibody, 1:4000, Invitrogen, Rockford, IL), calnexin (rabbit polyclonal antibody, 1:1000, Stressgen Bioreagents, Belgium), or the Na⁺-K⁺ ATPase α-1 subunit (mouse monoclonal antibody [a6F-s], 1:1000, Developmental Studies Hybridoma Bank) in TBST with 5% nonfat dry milk at 4°C. Membranes were washed 4 times with TBST for 10min each and incubated with secondary HRP-conjugated antibodies sheep anti-mouse (1:5000, Amersham, Piscataway, NJ) and goat anti-rabbit (1:10000, Jackson Immunoresearch, West Grove, PA) in TBST buffer with 3% nonfat dry milk at room temperature for 1hr. The blots were then washed 4 times for 10min each in TBST, and antibody interactions were detected with the ECL substrate (Pierce, Rockford, IL).

Cell Surface Biotinylation

HEK cells expressing SCN5A were washed with ice cold phosphate-buffered saline (PBS), and then treated with PBS containing 1.5 mg/ml sulfo-NHS-LC-biotin (Pierce) at 4 °C for

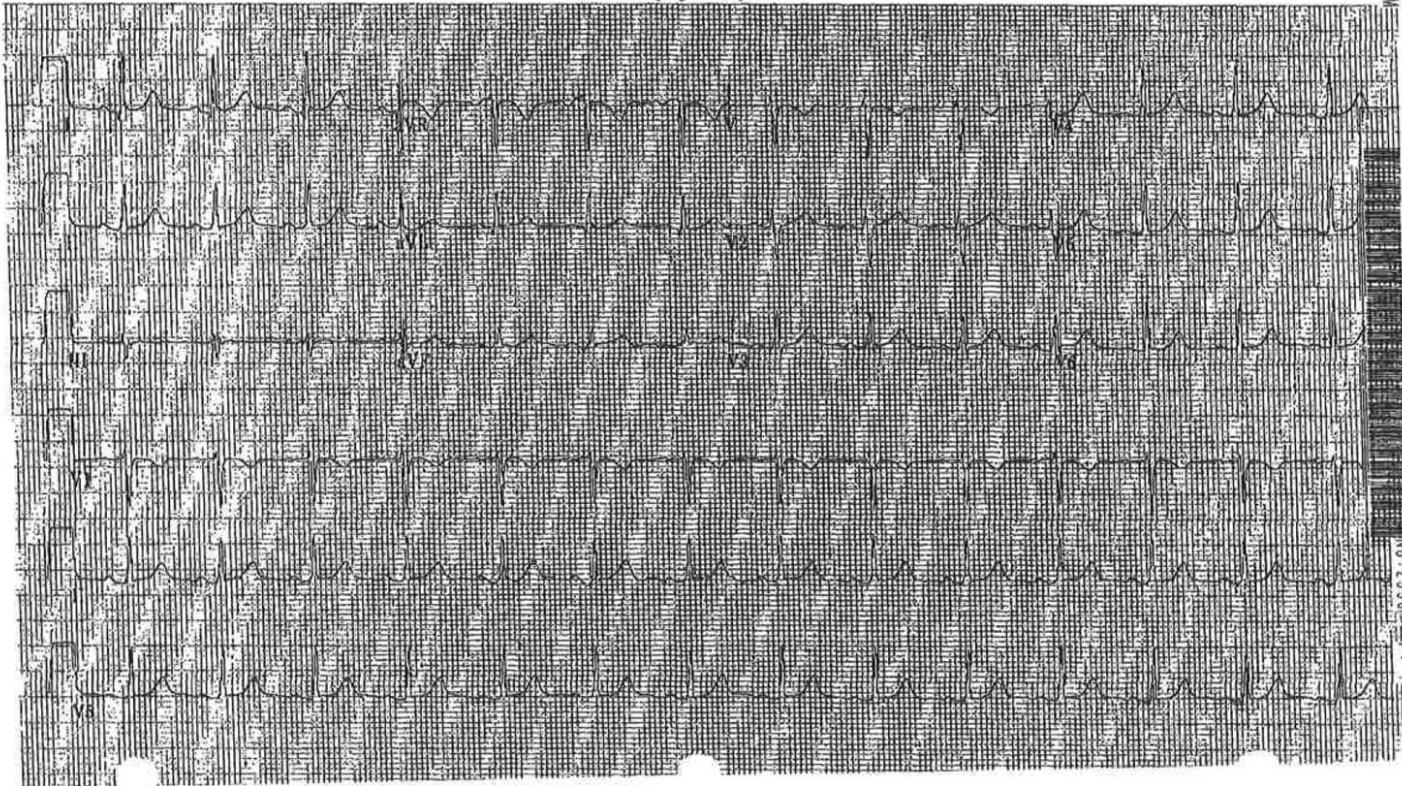
1hr. Excess biotin was inactivated and removed by a 20min incubation in 10 mM glycine/PBS at 4 °C and three washes in PBS. The cells were lysed in lysis buffer containing (in mM) 10 Tris (pH 7.3), 150 NaCl, 1 EDTA, 1% Triton X-100, and 1% sodium deoxycholate and a mixture of mammalian protease inhibitors (Roche). Cell lysates were spun at 13,000 × g for 20min, and the supernatants were incubated with streptavidin-coated beads (Pierce) for 1hr at room temperature. Beads were washed four times with lysis buffer and resuspended in 1× SDS-PAGE loading buffer, boiled, and analyzed by Western blot analysis.

FIGURES

ID: 427535585 23-Jan-2007 14:02:08
24years Vent. rate 85 bpm Normal sinus rhythm
Female PR interval 148 ms Normal ECG
QRS duration 80 ms
Room: er28 QT/QTc 380/452 ms
Loc: 0 P-R-T axes 26 22 28

Secondary ID: 0702300439
Referred by: johnson, s

Order no.: 30926
Unconfirmed



Jan. 24. 2007 11:38AM

Figure 1: ECG of the patient on admission. Episodes of polymorphic ventricular tachycardia (Figure 1 in the main paper) were recorded the following day.

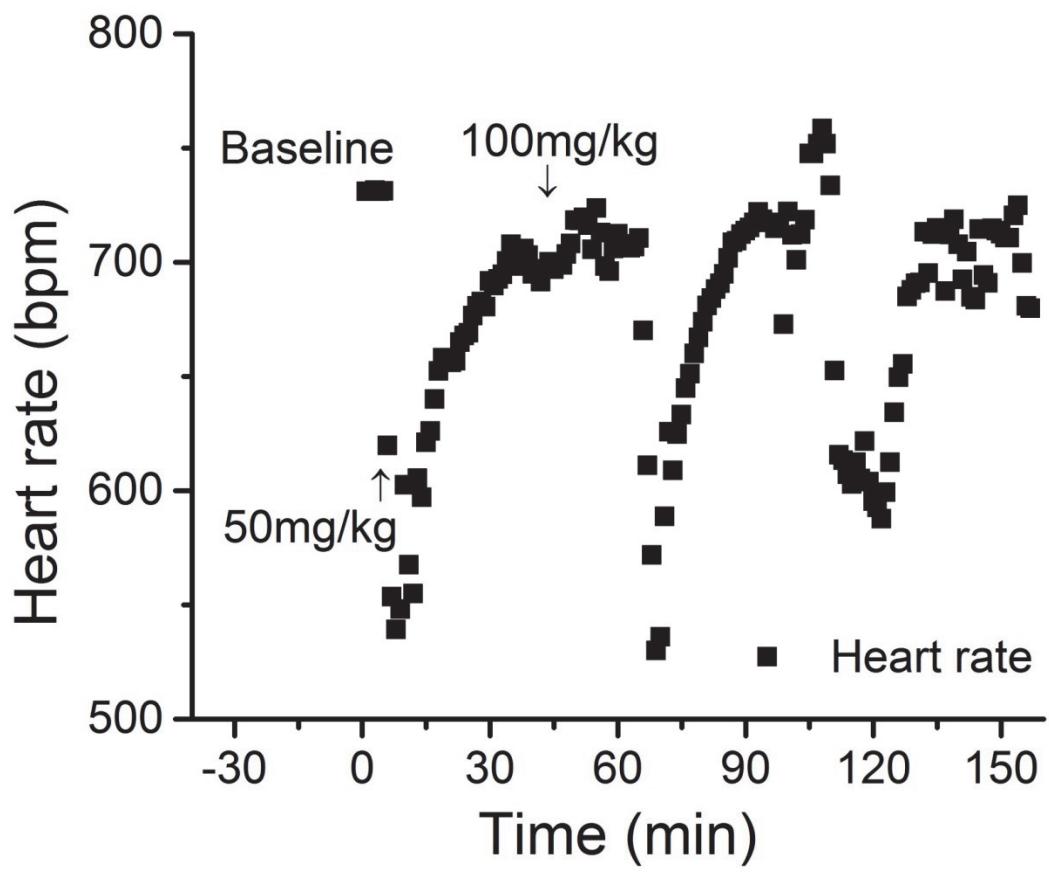
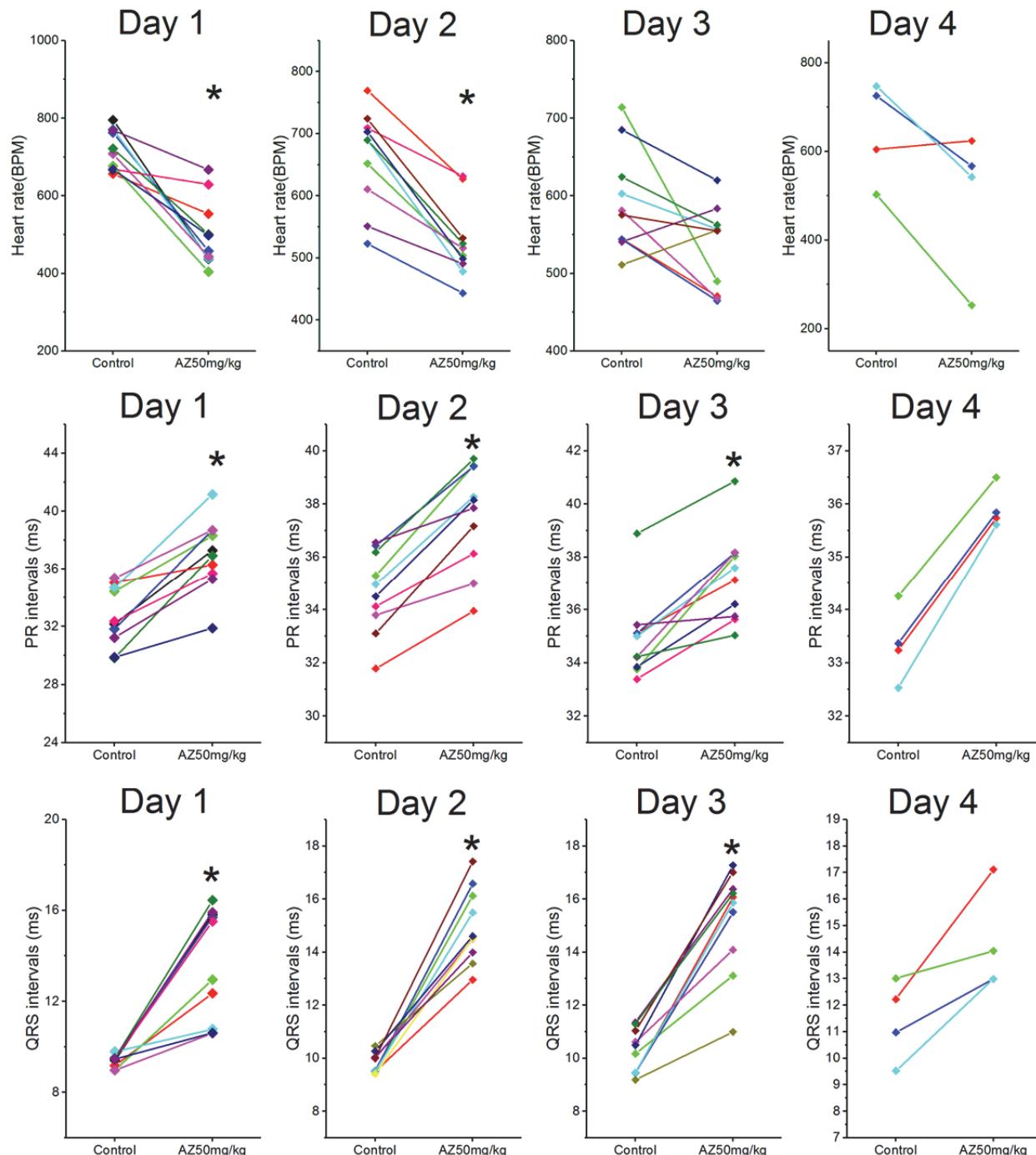


Figure 2: Reversible effects on IP azithromycin on sinus node automaticity/heart rate in a conscious telemetered mouse.



(Figure 3, continued on the next page)

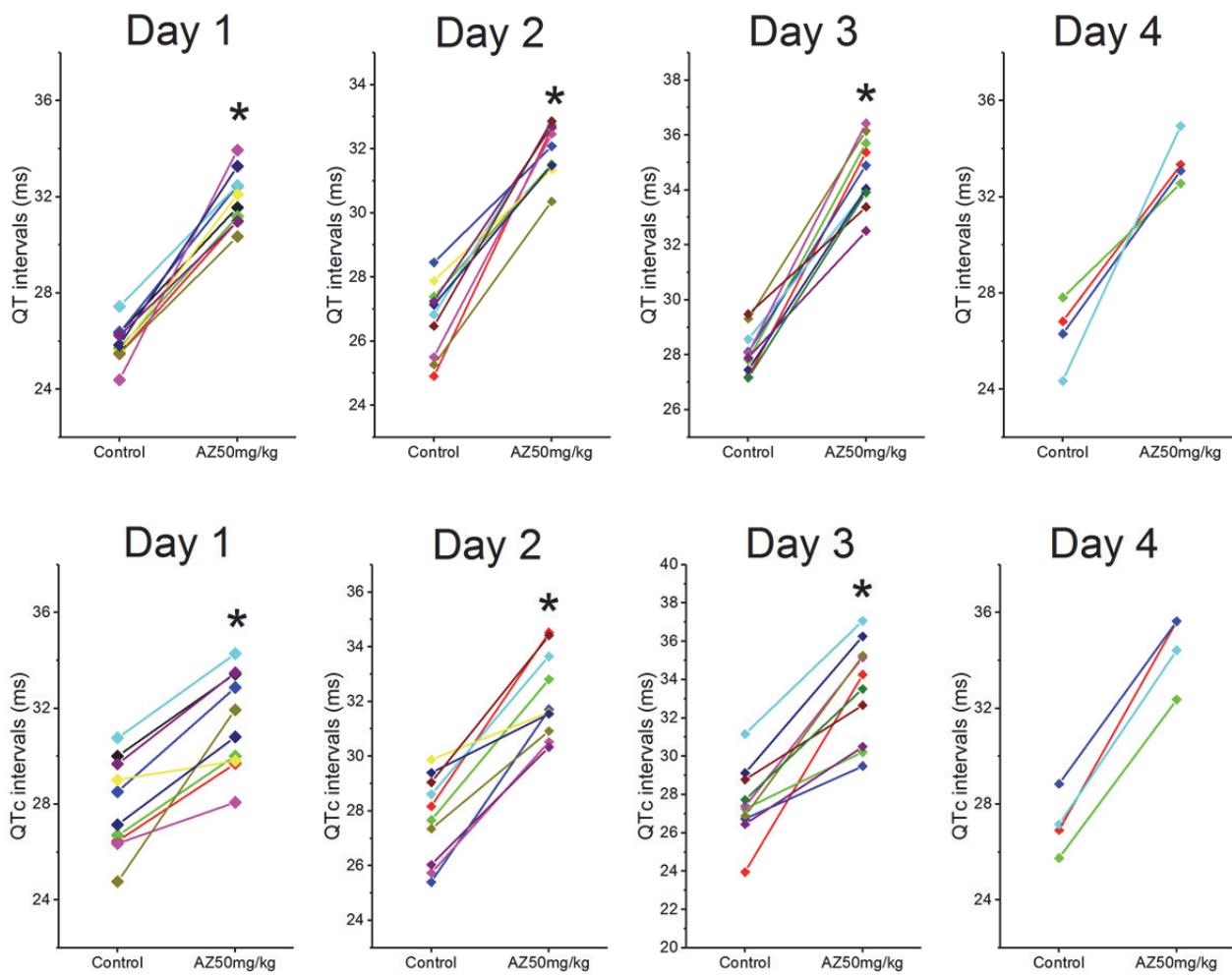


Figure 3: Effects of oral azithromycin (AZ) on the electrocardiogram of conscious adult mice. Mice received azithromycin (50 mg/kg/day) by oral gavage for 3 days, with baseline recording followed by additional recording for 2h post dose. To obtain samples to measure peak plasma concentrations, 4 mice were dosed on day 4. Representative measurements of heart rate are shown in the upper panel, with parameter data for the group shown below (n=10). With each dose of AZ, heart rate declined, along with an increase in the PR, QRS, and QT/QTc intervals (see Supplemental Table 2; *P<0.05), with small but progressive effects on trough or pre-dose values. Each mouse is represented by one color in all panels.

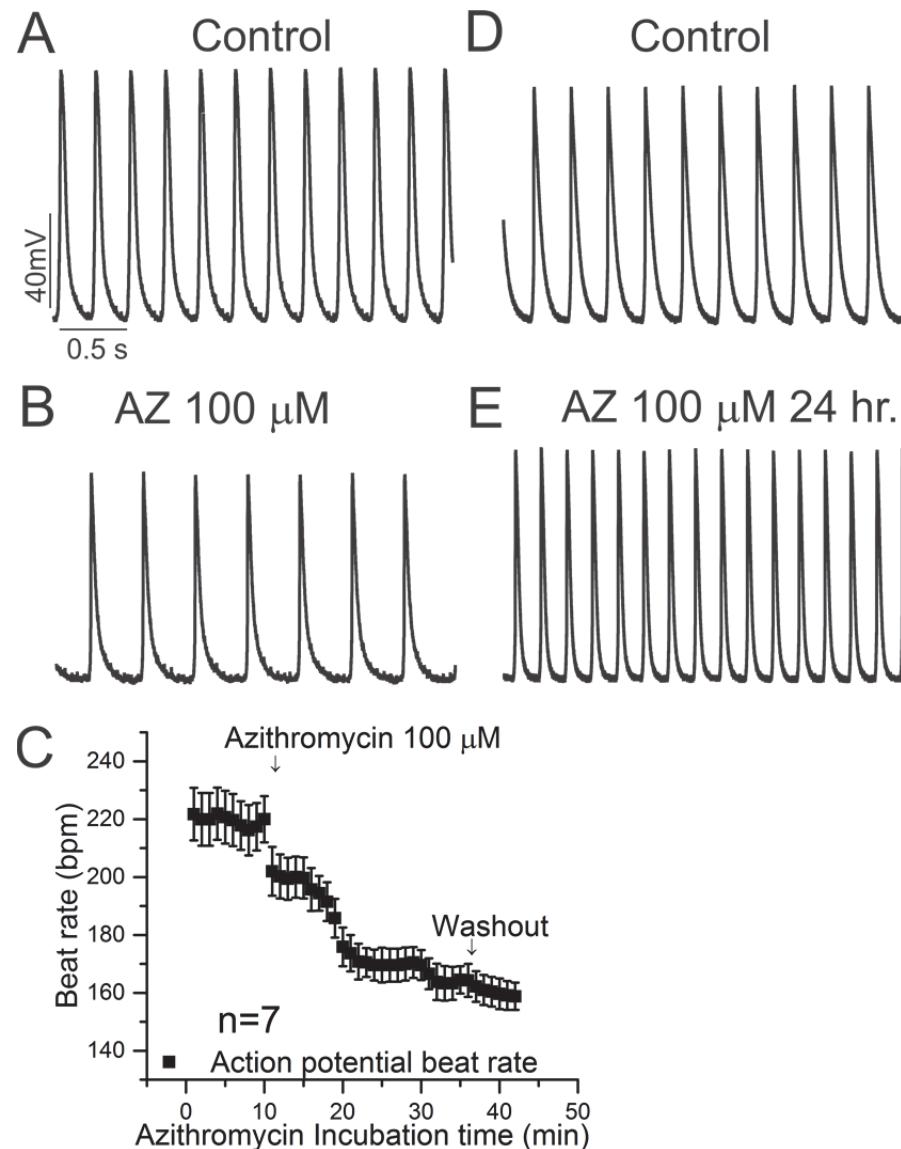


Figure 4: Effects on HL-1 cell automaticity. Compared to control conditions (**A**), acute exposure of HL-1 cells to AZ (100 μ M; 25 min) significantly slowed spontaneous beat rate (**B**). (**C**) Summary data for the time course of automaticity slowing is shown. **D** and **E**. Similar data are shown before (**D**) and after (**E**) a 24hr exposure to azithromycin.

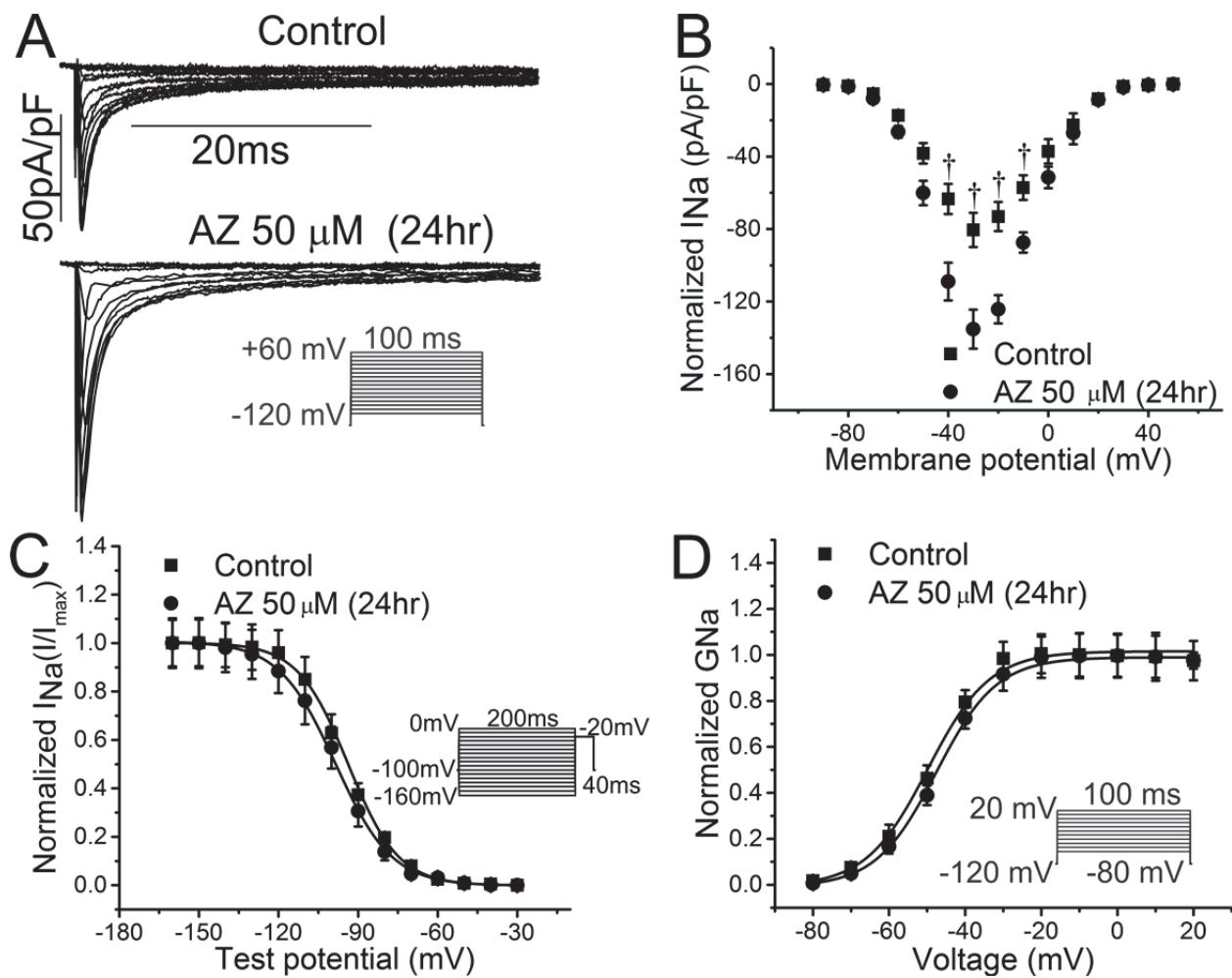
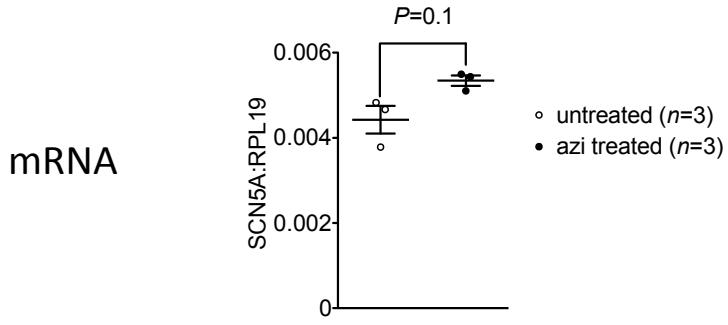
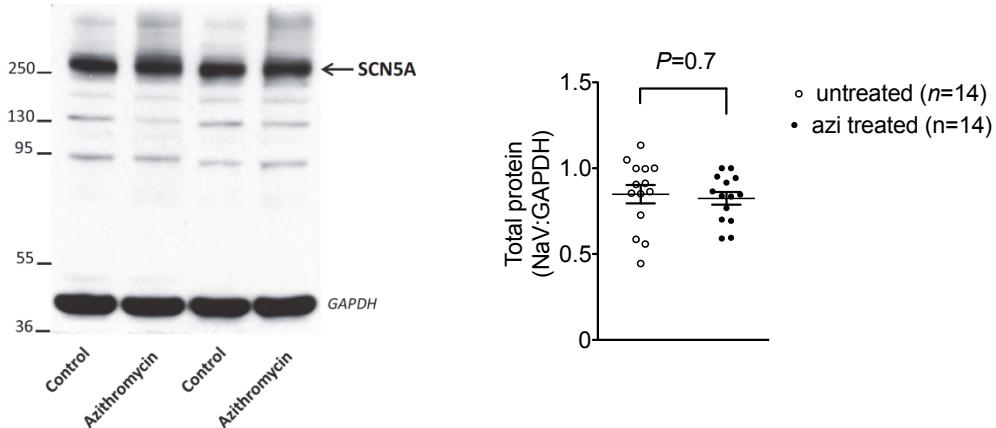


Figure 5: Increased peak Na^+ current in cardiomyocytes with chronic AZ exposure. **A** and **B**. In HL-1 cells, incubation with AZ (50 μM) for 24 hr significantly increased I_{Na} (from -81 ± 19 pA/pF to -135 ± 22 pA/pF at -20 mV; ${}^{\dagger}P < 0.01$). **C** and **D**. In the same preparation, similar shifts were observed in the steady-state inactivation and activation curves, as those seen with acute exposure (for inactivation, -93.8 ± 1.3 mV to -98.6 ± 1.4 mV for control and AZ 50 μM , respectively; for activation, -49.8 ± 1 mV to -47.6 ± 1 mV; $*P < 0.05$ for both).



Total cellular protein



Plasma membrane protein

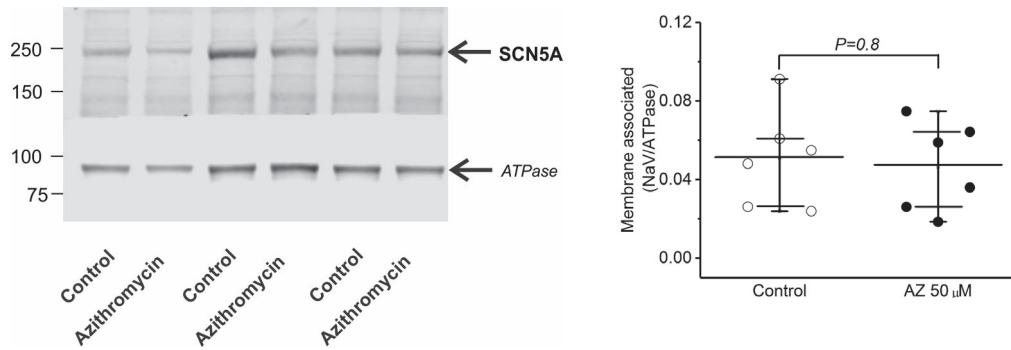


Figure 6: Lack of effect of chronic azithromycin exposure on mRNA, total cellular protein, and plasma membrane protein for SCN5A. Compared to control conditions, a 24hr exposure of HEK cells expressing SCN5A did not significantly change levels of mRNA (**top panel**), total cellular protein (**middle panel**), or SCN5A protein at the cell surface (**lower panel**, n=6).

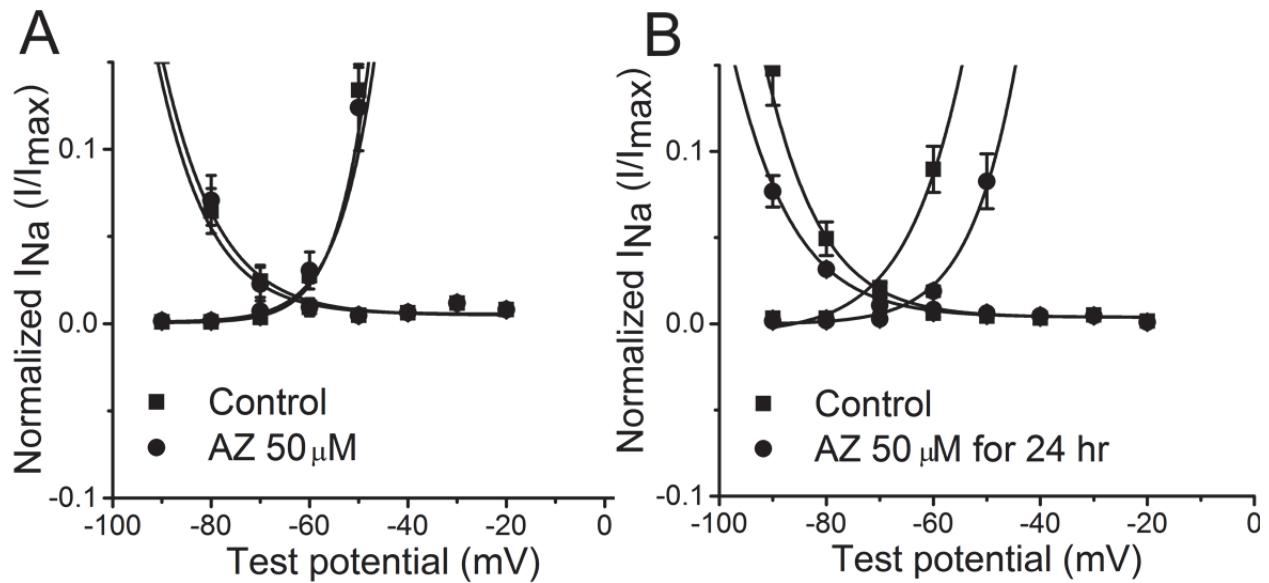


Figure 7: Minimal effects of acute and a 24hr exposure to azithromycin on SCN5A window currents.

A. Compared to control conditions, acute exposure of HEK cells expressing SCN5A to AZ (50 μ M; 25 min) had no effect on the area encompassed by overlap between activation and inactivation curves. **B.** With a 24hr exposure, azithromycin caused a small decrease in the SCN5A window current.

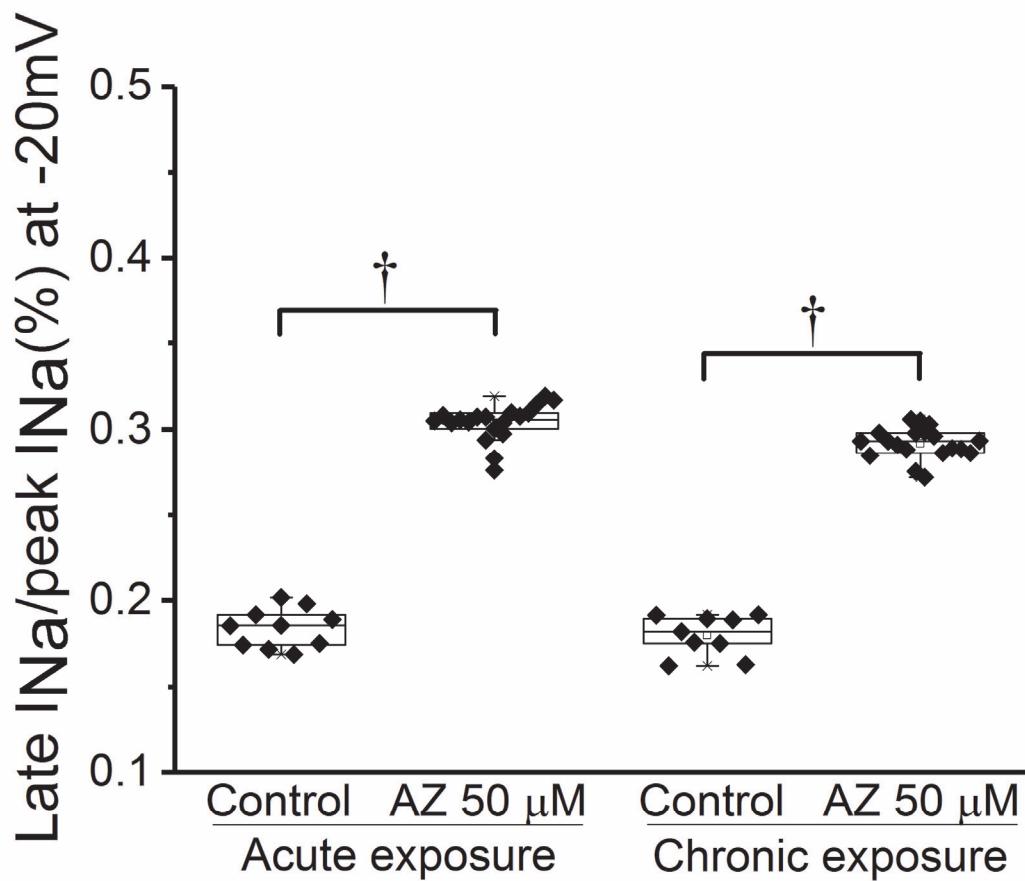


Figure 8: Effects of azithromycin following acute or 24hr exposure on the ratio of late I_{Na} /peak I_{Na} for SCN5A currents. For acute exposure, peak I_{Na} declined causing the ratio to increase, while for chronic exposure, the increase in late I_{Na} exceeded that for peak Na^+ current.

[†] $P<0.01$

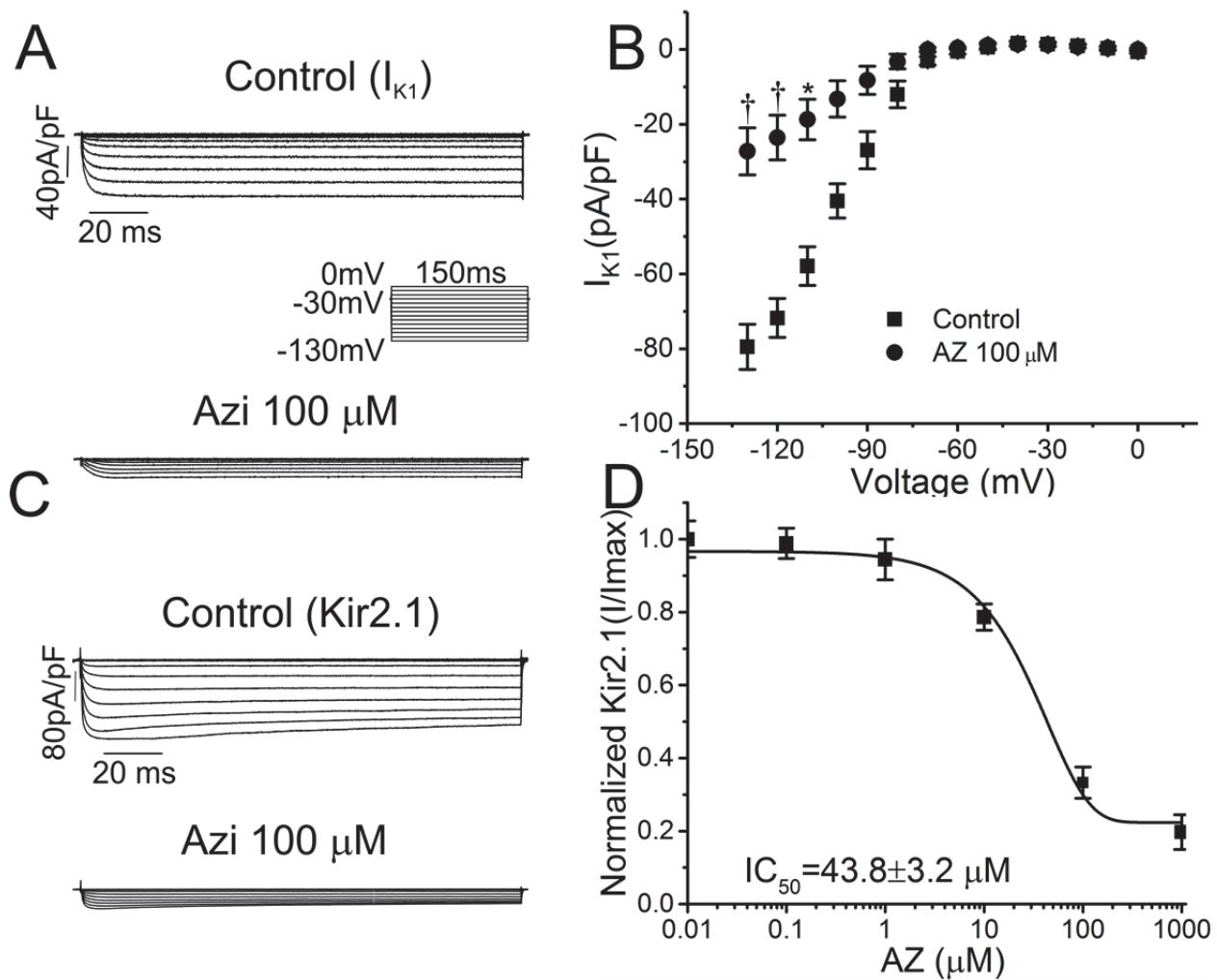


Figure 9: Effects of azithromycin on Kir2.1 currents in HEK cells and I_{K1} in HL-1 cell cardiomyocytes. **A, B, and C.** Acute exposure to azithromycin suppressed both I_{K1} currents in HL-1 cells (**A** and **B**), as well as K^+ currents derived from expression of human Kir2.1 in HEK cells (**C**). The IC_{50} for suppression of Kir2.1 currents was $43.8 \pm 3.2 \mu\text{M}$ (**D**). * $P < 0.05$; † $P < 0.01$

TABLES

Table 1: Estimated mean ECG parameter differences between treatment and baseline and their confidence intervals using linear regressions

	n	Estimated difference	Lower 95%	Upper 95%	P-value
Heart rate	21	10.3	8.1	12.4	<0.001
		30.2	27.7	32.7	<0.001
PR interval	21	2.8	1.1	4.6	0.004
		5.5	3.7	7.3	<0.001
QRS interval	21	3.2	2.8	3.6	<0.001
		4.9	4.3	5.5	<0.001
RR interval	21	10.3	8.1	12.4	<0.001
		30.2	27.7	32.7	<0.001
QT interval	21	6.2	4.6	7.8	<0.001
		8.5	6.4	10.6	<0.001
QTc interval	21	15.1	8.9	21.2	<0.001
		12.8	5.7	19.9	0.001

The first line is for the comparison between 50mg/kg and baseline. The second line is for the comparison between 100mg/kg and baseline.

Table 2: Effects of oral azithromycin on the ECG of conscious mice

		Heart rate (bpm)	PR (ms)	QRS (ms)	QT (ms)	QTc (ms)
Day 1	Control	720±16	32.7±0.7	9.4±0.1	25.9±0.3	27.9±0.6
	n=10 AZ 50mg/kg	503±28*	37.0±0.8*	13.7±0.8*	31.9±0.4*	31.4±0.6*
Day 2	Control	662±25	34.7±0.5	9.8±0.1	26.7±0.4	27.7±0.5
	n=10 AZ 50mg/kg	524±19*	37.5±0.6*	15.0±0.4*	32.0±0.3*	32.2±0.5*
Day 3	Control	592±21	34.9±0.5	10.2±0.3	28.1±0.3	27.5±0.6
	n=10 AZ 50mg/kg	533±17	37.3±0.5*	15.2±0.6*	34.6±0.4*	33.4±0.8*
Day 4	Control	645±57	33.3±0.4	11.4±0.8	26.3±0.7	27.2±0.6
	n=4 AZ 50mg/kg	497±83	35.9±0.2	14.3±1.0	33.5±0.5	34.5±0.8

QTc = QT/(RR/100)^{1/2}; *adjusted P<0.05

Table 3: Serum and cellular concentrations of azithromycin in humans (Zmax package insert)

Preparation	Zmax	3-day	5-day	Fold ↑
Daily dose (mg/kg)	28.6	7.14	3.6 (last 4 days)	
C_{max} ($\mu\text{g/ml}$) (μM)	0.82 1.1	0.44 0.59	0.43 0.58	
Concentration in WBC/PMN (μM)	146 195			178
Concentration in WBC/MN (μM)	116 155			141
Concentration in lung/alveolar cell (μM)	669 893			815

C_{max} is maximal concentration; WBC/PMN is polymorphonuclear leukocytes; WBC/MN is monocyte white blood cells.

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