Na⁺ channel distribution and electrophysiological heterogeneities in guinea pig ventricular wall

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Osadchii OE, Soltysinska E, Olesen SP. Na⁺ channel distribution and electrophysiological heterogeneities in guinea pig ventricular wall. *Am J Physiol Heart Circ Physiol* 300: H989–H1002, 2011. First published December 24, 2010; doi:10.1152/ajpheart.00816.2010.—We sought to explore the distribution pattern of Na⁺ channels across ventricular wall, and to determine its functional correlates, in the guinea pig heart. Voltage-dependent Na⁺ channel (Nav) protein expression levels were measured in transmural samples of ventricular tissue by Western blotting. Isolated, perfused heart preparations were used to record monophasic action potentials and volume-conducted ECG, and to measure effective refractory periods (ERPs) and pacing thresholds, in order to assess excitability, electrical restitution kinetics, and susceptibility to stimulation-evoked tachyarrhythmias at epicardial and endocardial stimulation sites. In both ventricular chambers, Na⁺ protein expression was higher at endocardium than epicardium, with midmyocardial layers showing intermediate expression levels. Endocardial stimulation sites showed higher excitability, as evidenced by lower pacing thresholds during regular stimulation and downward displacement of the strength-interval curve reconstructed after extrasystolic stimulation compared with epicardium. ERP restitution assessed over a wide range of pacing rates showed greater maximal slope and faster kinetics at endocardial than epicardial stimulation sites. Flecainide, a Na⁺ channel blocker, reduced the maximal ERP restitution slope, slowed restitution kinetics, and eliminated epicardial-to-endocardial difference in dynamics of electrical restitution. Greater excitability and steeper electrical restitution have been associated with greater arrhythmic susceptibility of endocardium than epicardium, as assessed by measuring ventricular fibrillation threshold, inducibility of tachyarrhythmias by rapid cardiac pacing, and the magnitude of stimulation-evoked repolarization alternans. In conclusion, higher Na⁺ channel expression levels may contribute to greater excitability, steeper electrical restitution slopes and faster restitution kinetics, and greater susceptibility to stimulation-evoked tachyarrhythmias at endocardium than epicardium in the guinea pig heart.

sodium channels; transmural heterogeneities; tachyarrhythmia

**METHODS**

The present study complies with the European Community Guidelines for the Care and Use of Experimental Animals and was approved by the Animal Ethics Screening Committee of the Panum Institute (clearance no. 2007/561-1341). Male Dunkin-Hartley guinea pigs
weighing 400–500 g were allowed to acclimatize to the housing conditions with free access to food and tap water for at least 7 days before entry into the study. On the experimental day, guinea pigs were anesthetized with pentobarbital sodium (50 mg/kg ip) and anticoagulated with heparin (1,000 IU/kg ip). The chest was opened, and the hearts were immediately excised and used either for tissue sampling to measure voltage-dependent Na+ channel (Na+) protein expression levels or for functional studies to assess electrophysiological properties of ventricular epicardium and endocardium in retrogradely perfused preparations.

**Western blotting.** Na+, protein expression levels were measured in thin samples of epicardial, endocardial, and midmyocardial tissue obtained from the base of the LV and right ventricular (RV) free wall with a pair of fine dissection scissors. Before ventricular tissue dissections, the hearts were briefly perfused with physiological saline solution using a Langendorff setup as described below to wash out the blood cells and ensure that cardiac tissue was not contaminated with serum proteins. The tissue was then snap-frozen in liquid nitrogen and stored at −80°C until further processing.

Total membrane and cytosolic proteins were prepared by using a method modified after Han et al. (32). Briefly, the snap-frozen tissues was quickly homogenized with the Precellys 24 system (Bertin Technologies) in 400 μl of cold buffer containing 20 mM Tris and 1 mM EDTA supplemented with a cocktail of protease inhibitors (10 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.2 μM leupeptin, 0.4 μM bestatin, 0.15 μM peptatin A, 0.14 μM M E-64, and 8 μM aprotonin; all from Sigma), Triton X-100 1% was added to solubilize membranes. The tissue suspension was left on ice for 2 h and then spun down (20,000 g, 10 min, 4°C). Supernatant was retained, and protein concentration was measured by the Lowry method (DC Protein Assay, Bio-Rad).

Proteins (50 μg/lane) were separated on precast 4–15% SDS-PAGE gels (Bio-Rad), transferred onto Hybond-P polyvinylidene fluoride transfer membranes (Amersham Biosciences), blocked in 5% nonfat milk in Tris-buffered saline-Tween 20 (10 mM Tris, 150 mM NaCl, pH 7.4) for 1 h at room temperature, and then incubated with Na+ channel antibody directed against all known vertebrate isoforms of Na+ protein (1:500, Sigma-Aldrich, clone K58/35) at 4°C overnight. To ensure antibody specificity for Na+ channel, the total lysate of nontransfected HEK293 cells transiently transfected with a human clone of Na+, 1.5 in pcDNA3 was used as a positive control. The total lysate of nontransfected HEK293 cells was used as a negative control.

The proteins were detected by horseradish peroxidase (HRP)-conjugated donkey anti-mouse antibody (1:10,000, Jackson ImmunoResearch Laboratories) and visualized by enzymatic chemiluminescence staining (Pierce). Immunoblots were exposed (for 2–3 min) to audioradiography film (Amersham Biosciences). To control for equal loading, the membranes were stripped in Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) and reprobed with a mouse anti-GAPDH antibody (MAB1501, Sigma-Aldrich). Band density (optical density × area) was quantified by Quantity One software (Bio-Rad) from films exposed equally.

**Isolated, Langendorff-perfused heart preparations.** The hearts were mounted on a Langendorff perfusion setup (Hugo Sachs Elektronik-Harvard Apparatus). The aortic perfusion pressure was measured with an ISOTEC pressure transducer connected to the aorta block of the setup. The ultrasonic flowmeter probe (Transonic Systems) and the thermocouple microprobe (Harvard Apparatus) were placed just above the aortic cannula to monitor the coronary flow rate (10–15 ml/min) and the temperature of the perfusion solution (37 ± 0.2°C), respectively. The electrical activity of the heart preparations was assessed from the volume-conducted ECG as well as monophasic action potential (MAP) recordings. Throughout the experiments, the heart preparations were kept immersed in the temperature-controlled, perfusate-filled chamber to enable ECG recording and to minimize thermal loss. Aortic pressure, coronary flow rate, ECG, and ventricular MAPs were continuously monitored with the 16-channel PowerLab system (ADInstruments, Oxford, UK).

**ECG.** A platform carrying four Ag/AgCl electrodes was placed in the perfusate-filled chamber just below the heart preparation to record volume-conducted ECG. The average QRS and QT interval values were determined from simulated standard leads I, II, and III and unipolar leads aVL, aVR, and aVF.

**Ventricular monophasic action potential.** MAPs were recorded with spring-loaded pressure contact electrodes (Hugo Sachs Elektronik-Harvard Apparatus). Three evenly spaced MAP electrodes were attached to the basal surface of the LV epicardium, and three MAP electrodes were placed on the RV epicardial surface. In a subset of experiments (n = 9), endocardial MAP was recorded at the LV anterior wall with an electrode advanced inside the LV via the AV orifice. The amount of pressure on the electrodes was adjusted to enable stable MAP recordings with an amplitude of at least 10 mV, smooth repolarization course, reproducible duration, and flat diastolic intervals. Both ECG and MAP signals were amplified, filtered at high cutoff (1 kHz) and digitized at a sampling frequency of 5 kHz (ADInstruments). The MAP duration was determined at 90% repolarization (APD90) during steady-state pacing and programmed ventricular stimulation and at 60% repolarization (APD60) during repolarization alternans. The mean epicardial APD90 was found as the average of MAP durations determined at six distinct ventricular recording sites. RV-to-LV transepicardial dispersion of repolarization was assessed as the difference between the maximal and minimal epicardial APD90 values. Transmural dispersion of repolarization was assessed as the difference between APD90 values measured at endocardial and opposite LV epicardial recording sites at the LV anterior wall.

**Electrical stimulations.** The stimulation protocols were applied to basal epicardial and endocardial stimulation sites in each ventricular chamber. Epicardial stimulations were performed with custom-made bipolar needle electrodes, and endocardial stimulations were accomplished with a bipolar stick-shaped stimulating electrode advanced into the ventricular chamber via a small incision made in the atrium. Heart preparations were stimulated with 2-ms rectangular pulses of negative polarity using the programmable stimulator (Hugo Sachs Elektronik-Harvard Apparatus). Assessments of arrhythmic susceptibility, ERP restitution, and spatial repolarization gradients were accomplished after stimulations at twice diastolic threshold current. Measurement of VF thresholds and assessments of ventricular excitability by testing the strength-interval relations were performed with a wide range of current intensities, as indicated below.

The arrhythmic susceptibility was assessed by appropriate stimulation protocols applied to intact (sinus node driven) heart preparations. In experiments designed to assess strength-interval relations, ERP restitution, or spatial repolarization gradients, both atria were removed and the AV node was crushed mechanically with forceps to slow down the intrinsic beating rate and therefore enable pacing over a wide range of S1–S2 intervals.

**Ventricular excitability.** The excitability at epicardial and endocardial stimulation sites was assessed by measuring pacing thresholds during regular stimulation as well as by reconstructing the strength-interval curves following premature extrasystolic stimulation. During regular stimulation, pacing thresholds were defined as the minimum current required for reproducible capture of a 20-beat drive train delivered at an interpulse (S−S2) interval of 250 ms. To reconstruct the strength-interval curves, the cardiac cycle was scanned by application of extrastimulus (S2) at progressively decreasing coupling interval after the burst of 10 regular (S1) pulses delivered.
at an $S_1-S_1$ interval of 500 ms. The measurements were started with a coupling stimulation ($S_1-S_2$) interval of 450 ms, which then was reduced in 50-ms steps to 200 ms and thereafter with a decrement of 3–10 ms until the refractoriness was reached. At each $S_1-S_2$ interval tested, we measured the minimal current strength at which the extrastimulus was able to elicit a propagating response. The increment in stimulating current strength upon consecutive $S_1-S_2$ interval shortenings was repeated until the current applied reached 10 diastolic threshold values (1.0–1.5 mA for endocardial stimulations and 5.0–8.0 mA for epicardial stimulations). The strength-interval relationships were analyzed by plotting the measured threshold current strength as a function of $S_1-S_2$ interval, and the data were fit to a hyperbolic function.

**Ventricular fibrillation threshold.** The minimal current strength inducing VF was determined with a burst of 50 pulses ($S_1-S_1$ interval of 20 ms) applied at progressively increasing current intensities starting from an initial value of 5 mA. In successive stimulations, the current intensity was increased with an increment of 1 mA until VF was induced. To allow repeated measurements, VF episodes persisting longer than 20 s were terminated by bolus injection of highly concentrated KCl solution (2 ml, 50 mM) just above the aortic cannula.

**Rapid cardiac pacing.** To assess susceptibility to repolarization alternans and VF inducibility at epicardial and endocardial stimulation sites, a burst of 100 pulses (current intensity of twice diastolic threshold) was applied at progressively increasing pacing rates. For this purpose, the interpulse pacing interval in successive stimulations was reduced by 10 ms starting from the initial value of 250 ms. The protocol was continued until VF was induced or the minimum value of interpulse interval (10 ms) was achieved. The repolarization alternans preceding VF induction was defined as regular beat-to-beat oscillations in action potential duration where the APD$_{50}$ difference in each pair of long-short action potentials was ≥5 ms.

**Ventricular extrasystolic stimulation.** The LV was paced at either the epicardial or the endocardial stimulation site at an $S_1-S_1$ interval of 250 ms, and the premature extrasystimulus ($S_2$) was applied after each tenth regular pulse at progressively reducing coupling stimulation intervals. The stimulations were started with an $S_1-S_2$ interval of 240 ms, which was then reduced with a decrement of 10 ms until the refractoriness was reached.

**Restitution of effective refractory period.** Modeling studies suggest that reduction in $I_Na$ density or slowing its recovery from inactivation produces little effect on action potential duration while significantly prolonging the ERP (56). Consistently, only a small (by 10%) APD$_{50}$ shortening due to inhibition of the late $I_Na$ has been produced by tetrodotoxin in guinea pig ventricular myocytes (40). In the present study, we therefore considered that ERP rather than APD$_{50}$ changes are more representative to explore the relations between availability of Na$^+$ channels and electrical restitution kinetics.

The protocol of ERP restitution was implemented at ventricular epicardial and endocardial stimulation sites as described previously (51). The ERP was measured by an extrastimulus method whereby a burst of 15 regular ($S_1$) pulses was followed by an extrastimulus ($S_2$) applied at variable coupling stimulation intervals. The measurements were started with $S_1-S_1$ cycle length of 500 ms, which then was reduced in 20-ms steps to 200 ms and thereafter with a decrement of 5–10 ms until the capture was lost at pacing intervals of 140–160 ms. At all pacing cycle lengths used, the ERP was defined as the longest $S_1-S_2$ interval at which a premature extrastimulus failed to elicit a propagating response.

Once the stimulation protocol was completed, appropriate diastolic intervals (DIs) were calculated as a difference between the $S_1-S_1$ pacing cycle length used and the ERP measured. ERP was then plotted as a function of the preceding DI, and the restitution curves were fitted with the double-exponential function: $y = y_0 + A_1e^{-DI/\tau_1} + A_2e^{-DI/\tau_2}$, where $y$ represents ERP, $y_0$ is a free-fitting variable, and $A_1$ and $A_2$ are the amplitudes and $\tau_1$ and $\tau_2$ the time constants of the fast ($A_1$ and $\tau_1$) and slow ($A_2$ and $\tau_2$) exponential components obtained by a least-squares fit. The curve fitting was performed with Igor Pro 6.0 software (WaveMetrics, Portland, OR).

In each experiment, the ERP restitution curves were analyzed to determine the maximal restitution slope and the range of ERP changes (ΔERP, the maximum-to-minimum change) yielded by the stimulation protocol. The maximal slope of ERP restitution was found by analyzing the first derivative of exponential fit. The electrical restitution kinetics was assessed by an empirical rate constant (44) calculated as a ratio between $\Delta$ERP and the range of DIs covered by the stimulation protocol (ΔDI, the difference between the longest and the shortest DIs).

**Pharmacological studies.** The impact of drug-induced Na$^+$ channel blockade on epicardial and endocardial ERP restitution was assessed after infusion of flecainide (Sigma-Aldrich) at a dose of 1.5 μM. For precise flecainide dosing, the heart preparations were perfused with physiological saline solution as described above at a constant flow of 15 ml/min, and flecainide was infused over 30 min at a rate of 0.3 ml/min with a calibrated infusion pump.

**Data analysis.** Data analysis was performed with Chart 5-Pro for Windows software (ADInstruments). Data are expressed as means ± SE. Na$^+$ protein expression levels in transmural samples of ventricular tissue were compared by repeated-measures ANOVA followed by Bonferroni post hoc test. Paired $t$-tests were used to compare variables determined at ventricular epicardial and endocardial stimulation sites, both at baseline and after flecainide infusion. The inducibility of VF by tachypacing at epicardium and endocardium was assessed with Fisher’s exact test. $P$ values < 0.05 were considered to be significant.

**RESULTS**

Na$^+$ protein expression levels across ventricular wall. A representative Western blot showing transmural Na$^+$ protein expression in LV and RV is shown in Fig. 1A, and the composite data are shown in Fig. 1B. In both ventricular chambers, Na$^+$ protein expression levels were found to show nonuniform distribution across ventricular wall, with the highest levels determined in endocardial layers and the lowest in epicardial layers. A specific band at the expected molecular mass of ~230 kDa was detected in Na$^+$/HEK293 cells but not in nontransfected HEK293 cells.

**Ventricular excitability.** Consistent with greater Na$^+$ protein expression, ventricular endocardium showed greater excitability than epicardium. In particular, ventricular pacing thresholds measured at a constant ($S_1-S_1 = 250$ ms) stimulation interval were significantly lower at endocardial than epicardial stimulation sites in the LV [epicardial (Epi, $n = 12$): 0.63 ± 0.04 mA, endocardial (Endo, $n = 12$): 0.16 ± 0.04 mA; $P < 0.0001$] and the RV [Epi ($n = 12$): 0.57 ± 0.04 mA, Endo ($n = 12$): 0.13 ± 0.02 mA; $P < 0.0001$].

The strength-interval curves were reconstructed with a premature extrasystolic stimulation protocol to compare excitability at epicardial and endocardial stimulation sites over a wide range of time points throughout the cardiac cycle (Fig. 2). With both epicardial and endocardial stimulations, the excitation thresholds remained unchanged over flat diastolic portion of the excitability curve but were incrementally enhanced with increasing extrastimulus prematurity over a range of short ($S_1-S_2 < 190$ ms) coupling stimulation intervals close to the absolute refractory period, thereby yielding the hyperbolic shape of the strength-interval relations. In both ventricular chambers, the endocardial strength-interval curves were displaced downward along the y-axis, suggesting that a lower current strength was required to elicit a propagating response at
into an epicardial-to-endocardial difference in the shortest pacing interval that enables 1:1 ventricular capture upon progressive increase in beating rate. The minimal pacing intervals in sinus node-driven heart preparations were found to be significantly shorter at endocardial than epicardial stimulation sites in the LV [Epi (n = 12): 115 ± 5 ms, Endo (n = 12): 100 ± 5 ms; P = 0.04] and the RV [Epi (n = 12): 133 ± 5 ms, Endo (n = 12): 118 ± 4 ms; P = 0.009]. The minimal pacing intervals were shorter at LV than RV stimulation sites over ventricular epicardium (P = 0.02) and endocardium (P = 0.01).

Susceptibility to electrical stimulation-evoked tachyarrhythmias. Greater myocardial excitability and steeper ERP restitution slopes may contribute to higher arrhythmic susceptibility at endocardial than epicardial stimulation sites. VF thresholds were found to be lower at endocardial than epicardial stimulation sites in the LV [Epi (n = 12): 15 ± 2 mA, Endo (n = 12): 6 ± mA; P = 0.009] and the RV [Epi (n = 12): 15 ± 3 mA, Endo (n = 12): 9 ± 2 mA; P = 0.04]. Furthermore, in both ventricular chambers, the inducibility of tachyarrhythmias by rapid cardiac pacing was higher during endocardial than epicardial stimulation (Fig. 4, A–C). During endocardial stimulation, the VF induction at fast pacing rates was invariably preceded by repolarization alternans representing regular beat-to-beat oscillations in APD60 and the T-wave amplitude on volume-conducted ECG (Fig. 4B, fragments 3 and 4). The repolarization alternans was promoted at pacing intervals 10–30 ms longer than those inducing tachyarrhythmia. Over this range of pacing intervals, the progression from repolarization alternans to VF following further increase in ventricular comparable coupling stimulation intervals at endocardial than at epicardial stimulation sites (Fig. 2).

Restitution of effective refractory period. Nonuniform distribution of Na⁺ channels may contribute to differential changes in refractoriness at epicardial and endocardial sites upon progressive increase in cardiac beating rate. This issue has been addressed by exploring the ERP restitution. A reduction in preceding DI upon progressively increasing ventricular pacing rates has been followed by exponential shortening of epicardial and endocardial ERPs in the LV (Fig. 3A) and the RV (Fig. 3E). However, the ERP restitution slopes over a range of short (<100 ms) DIs (Fig. 3, B and F), as well as the maximal slope values (Fig. 3, C and G), were greater at endocardial than epicardial stimulation sites. The amplitude of the ERP restitution curve as measured by the maximum-to-minimum ERP change was greater at endocardial than epicardial stimulation sites in the LV [Epi (n = 9): 36 ± 3 ms, Endo (n = 9): 44 ± 2 ms; P = 0.01] and the RV [Epi (n = 9): 40 ± 2 ms, Endo (n = 9): 51 ± 3 ms; P = 0.01]. Consequently, the endocardial stimulation sites exhibited faster restitution kinetics as evidenced by greater restitution rate constant values in LV (Fig. 3D) and RV (Fig. 3H).

Minimal pacing intervals. We sought to determine whether greater endocardial Na⁺ protein expression levels may translate into a faster ERP restitution in endocardial sites. A significant reduction in ERP restitution slopes, as evidenced by greater ERP restitution rate constant values, was observed at endocardial stimulation sites in both the LV (P < 0.05) and RV (P < 0.05). ERP restitution rate constant values were significantly greater at endocardial than epicardial sites in the LV (P < 0.05) and RV (P < 0.05).
stimulation rate was associated with significant enhancement in the magnitude of the maximum-to-minimum APD$_{60}$ change in each pair of long-short action potentials (Fig. 4B, fragments 3 and 4). In ~40% of experiments, the repolarization alternans was also induced by tachypacing applied to the ventricular epicardial stimulation site (Fig. 4A, fragment 4), but in this case the magnitude of beat-to-beat oscillations in APD$_{60}$ was significantly lower than during endocardial stimulations (Fig. 4D). The overall data on repolarization alternans induced after epicardial and endocardial stimulations in each ventricular chamber are summarized in Table 1.

LV extrasystolic stimulation ($n = 19$) at coupling intervals of 5–10 ms greater than ERP duration elicited tachyarrhythmia in 26% of experiments when applied to endocardial stimulation sites but produced no VT during epicardial stimulations (Supplemental Material). Only short-lasting, self-terminating VTs [mean VT duration ($n = 5$): 1.2 ± 0.3 s] were elicited upon LV endocardial S$_1$-S$_2$ stimulations.

Spatial repolarization gradients after epicardial and endocardial pacing. Epicardial-to-endocardial difference in arrhythmic susceptibility has not been related to alterations in spatial repolarization gradients produced by changes in location of ventricular stimulation site (Fig. 5). Epicardial APD$_{90}$ values assessed at the longest pacing interval (S$_1$-S$_1$ = 550 ms) producing no spontaneous escaped beats were found to be greater at RV than LV recording sites during LV epicardial pacing [mean LV APD$_{90}$ ($n = 9$): 160 ± 2 ms, mean RV APD$_{90}$ ($n = 9$): 168 ± 3 ms; $P = 0.0007$] and LV endocardial pacing [mean LV APD$_{90}$ ($n = 9$): 161 ± 2 ms, mean RV APD$_{90}$ ($n = 9$): 168 ± 3 ms; $P = 0.002$]. At the LV anterior wall, the endocardial APD$_{90}$ values were 5–10 ms greater than APD$_{90}$ determined at opposite epicardial recording site (Fig. 5, D and E). Similar RV-to-LV transepidericardial APD$_{90}$ dispersion values (Fig. 5F) were found after LV epicardial and LV endocardial pacing.

Pharmacological responses to Na$^+$ channel blocker. In spontaneously beating heart preparations, flecainide infusion modestly increased the width of the QRS complex on ECG [basal ($n = 9$): 26 ± 1 ms, flecainide ($n = 9$): 30 ± 2 ms; $P = 0.009$] but had no effect on the cardiac cycle length [basal ($n = 9$): 570 ± 22 ms, flecainide ($n = 9$): 605 ± 25 ms; $P = 0.27$], mean ventricular epicardial APD$_{90}$ [basal ($n = 9$): 165 ± 2 ms, flecainide ($n = 9$): 169 ± 3 ms; $P = 0.1$], and LV endocardial APD$_{90}$ [basal ($n = 9$): 174 ± 4 ms, flecainide ($n = 9$): 179 ± 5 ms].

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1 Supplemental Material for this article is available online at the Journal website.
Fig. 4. Susceptibility of epicardial and endocardial stimulation sites to repolarization alternans and tachyarrhythmias following rapid cardiac pacing. A and B: representative changes of volume-conducted ECG (top trace in each fragment) and RV epicardial monophasic action potential (bottom trace in each fragment) following steady-state pacing at progressively increasing rates applied at RV epicardial (A) and RV endocardial (B) stimulation sites. Vertical dashed lines indicate the moments of pacing stimulus application. Horizontal solid lines and numbers (in ms) under the monophasic action potential traces show the pacing intervals used. Numbers given on right in fragment 4 in A and fragments 3 and 4 in B show action potential duration at 60% repolarization (APD_{60}) values (ms) in each pair of long-short action potentials during repolarization alternans. Ventricular pacing at S1-S1 of 80 ms (fragment 5 in A and B) resulted in 2:1 conduction block when applied at epicardial stimulation site but promoted tachyarrhythmia following endocardial stimulation. Note that shortening of the pacing interval results in increase in the magnitude of repolarization alternans before tachyarrhythmia induction at endocardial stimulation site (fragments 3 and 4 in B). Also note beat-to-beat changes in T-wave amplitude during APD_{60} alternans (fragment 4 in A and fragments 3 and 4 in B). C: numbers above bars reflect no. of experiments in which ventricular tachyarrhythmia (VT) was induced vs. total no. of experiments. D: maximal magnitude of APD_{60} oscillations determined in a subset of experiments in which tachypacing was found to induce repolarization alternans. *P < 0.05 vs. epicardium (C and D).

ms; P = 0.34]. The LV extrasystolic stimulations using the protocol shown in the Supplemental Material for this article revealed no proarrhythmic effects of flecainide at either epicardial or endocardial stimulation sites. The ERP was prolonged upon flecainide infusion both at LV epicardium [basal (n = 5): 120 ± 9 ms, flecainide (n = 5): 140 ± 9 ms; P = 0.04] and LV endocardium [basal (n = 5): 122 ± 10 ms, flecainide (n = 5): 143 ± 10 ms; P = 0.04].

Flecainide-induced changes in refractoriness were explored in more detail by analyzing the ERP restitution. Flecainide was found to lengthen ventricular ERPs over a wide range of DI, thereby causing the ERP restitution curve to shift upward (Fig. 6, A, D, G, and J). Consistently, the shortest pacing interval to enable ventricular capture was significantly increased by flecainide (Table 2). Flecainide flattened the ERP restitution curve both at epicardial and endocardial stimulation sites, as evidenced by decreased maximal ERP restitution slope values (Fig. 6, C, F, I, and L) and reduced amplitude of the maximum-to-minimum ERP change yielded by stimulation protocol (Table 2). The restitution rate constant values were markedly reduced in flecainide-treated heart preparations (Table 2), suggesting slowed epicardial and endocardial ERP restitution

<table>
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<tr>
<th>Table 1. Parameters of alternans of monophasic action potential duration and T-wave amplitude on ECG after LV and RV epicardial and endocardial tachypacing in guinea pig heart</th>
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<td>Pacing interval, ms</td>
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Values are means ± SE for parameters of the alternans of monophasic action potential duration (APD) and T-wave amplitude on ECG following left ventricular (LV) and right ventricular (RV) epicardial (epi) and endocardial (endo) tachypacing in the guinea pig heart. APD_{60}, APD at 60% repolarization. *P < 0.05 vs. epicardial value.
Ventricular activation after epicardial and endocardial pacing. To reveal whether the epicardial-to-endocardial difference in excitability, electrical restitution, and arrhythmic susceptibility may be ascribed to stimulation of Purkinje fibers following endocardial pacing, we assessed ventricular activation in spontaneously beating and paced heart preparations by measuring the duration of the QRS complex on volume-conducted ECG (Fig. 7). Consistent with fast conduction via Purkinje fibers, the ventricular depolarization showed much shorter duration in intact spontaneously beating heart preparations than in AV-blocked heart preparations applied over a wide range of S1-S1 intervals to either the LV or the RV chamber (Fig. 7, B and C). These findings may suggest that conduction via ventricular contractile fibers rather than specialized Purkinje fibers plays a pivotal role in ventricular activation during both endocardial and epicardial pacing.

DISCUSSION

Main findings. In the present study, we found that Na⁺ channels are distributed nonuniformly across ventricular wall, with significantly greater Na⁺ protein expression levels determined at endocardial than epicardial layers. We also show that epicardial-to-endocardial difference in distribution of Na⁺ channels may account for transmural electrophysiological heterogeneities, including greater tissue excitability, steeper ERP restitution slopes and faster restitution kinetics, shorter minimal pacing intervals that allow 1:1 ventricular capture, and greater susceptibility to repolarization alternans and stimulation-induced tachyarrhythmias at endocardium than at epicardial pacing.
This is the first study to establish the link between nonuniform Na⁺/H₁⁻¹⁺ channel distribution and electrophysiological heterogeneities across ventricular wall.

Ventricular stimulation-induced arrhythmogenicity and Na⁺ channels. In cardiac patients, the RV endocardial apex is considered as the stimulation site of choice because it is easily accessible via the transvenous route and shows no pacemaker lead dislodgements and stable pacing thresholds over a long-term time frame (74). Nevertheless, endocardial pacing may be associated with sudden cardiac death due to malignant VT resulting from interference of pacemaker rhythm and spontaneous ventricular ectopic beats, which accounts for 23% of fatal outcomes in paced patients (86). Furthermore, pacemaker-induced VT has been detected in 26% of patients with an implantable cardioverter-defibrillator (34). VT inducibility by programmed stimulation as assessed after cardiac surgery is higher at RV endocardial than RV epicardial stimulation sites (70). Postoperative assessments of the efficacy of antitachyarrhythmia surgery suggest that epicardial stimulation implemented alone may underestimate the number of patients at risk for recurrent VT by 25%, thereby highlighting the important predictive value of endocardial testing (9). Taken together, these findings suggest that ventricular endocardial pacing may have proarrhythmic potential in a significant proportion of cardiac patients.

In animal studies, lower VF thresholds have been found at LV endocardium than epicardium (37). In canine hearts, chemical ablation of the endocardium has been found to lead to substantial increase in LV fibrillation threshold (22) and prevents degeneration of ischemia-induced ectopic rhythms into VF (38). Importantly, ventricular endocardium plays a pivotal role in maintaining electrical stimulation-induced VF, where

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Table 2. Effects of flecainide on ventricular pacing thresholds and parameters of ERP restitution in guinea pig heart

<table>
<thead>
<tr>
<th></th>
<th>LV Epicardium</th>
<th>LV Endocardium</th>
<th>RV Epicardium</th>
<th>RV Endocardium</th>
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<tr>
<td>Pacing threshold, mA</td>
<td>0.65 ± 0.08 0.78 ± 0.12</td>
<td>0.15 ± 0.02 0.40 ± 0.10*</td>
<td>0.51 ± 0.06 0.62 ± 0.14</td>
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<td>Minimal pacing interval, ms</td>
<td>169 ± 4 183 ± 6*</td>
<td>163 ± 3 188 ± 3*</td>
<td>163 ± 4 182 ± 4*</td>
<td>159 ± 4 188 ± 4*</td>
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<tr>
<td>Amplitude of ERP restitution curve, ms</td>
<td>35 ± 2 22 ± 5*</td>
<td>39 ± 1 24 ± 4*</td>
<td>40 ± 3 29 ± 5*</td>
<td>48 ± 2 30 ± 5*</td>
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<tr>
<td>Restitution rate constant</td>
<td>0.12 ± 0.01 0.08 ± 0.01*</td>
<td>0.14 ± 0.01 0.08 ± 0.01*</td>
<td>0.14 ± 0.01 0.10 ± 0.01*</td>
<td>0.17 ± 0.01 0.11 ± 0.02*</td>
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Values are means ± SE for n = 9 hearts/group. All variables were determined in heart preparations with removed atria and ablated atrioventricular node to enable pacing over a wide range of intervals. ERP, effective refractory period. *P < 0.05 vs. basal value.
most excitation wave fronts first originate at endocardium and then experience variable degrees of block while propagating to epicardium (84).

It is uncertain whether the epicardial-to-endocardial difference in arrhythmic susceptibility may be partly related to the nonuniform distribution of Na⁺ channels across ventricular wall and, if so, which mechanisms may account for these relations. Indeed, although transmural gradient in Na⁺ channel expression has been found in some studies (26, 59, 63, 72), its physiological importance and impact on arrhythmic susceptibility remain uncertain. Importantly, exploration of the functional correlates of heterogeneous Na⁺ channel distribution in human ventricular tissue is limited because of ethical constraints. In the present study, we used the guinea pig heart model to show greater endocardial than epicardial Na⁺ protein expression levels, which is consistent with our previous findings in failing and nondiseased human ventricular tissue (72). We also replicate clinical findings on greater proarrhythmic potential of endocardial than epicardial pacing (9, 70), as assessed by susceptibility to repolarization alternans and VT inducibility by rapid cardiac pacing, measuring VF threshold values following burst stimulation, and evaluating propensity to short-lasting tachyarrhythmia by extrasystolic stimulation. Furthermore, we explore the mechanisms staying behind these differences, to show that greater arrhythmic susceptibility to endocardial pacing may be related to greater excitability and steeper ERP restitution slopes at endocardium than epicardium.

**Epicardial-to-endocardial difference in excitability.** To assess excitability changes throughout the cardiac cycle at epicardium and endocardium, we analyzed the strength-interval relations reconstructed after bipolar stimulations with pulses of negative polarity. Previous experimental (3–4, 27, 42, 49, 67) and clinical (12, 31, 43) studies validated the utility of bipolar stimulation protocols for assessments of ventricular excitability changes after acute myocardial ischemia (3), stimulation of the autonomic nerves (27, 42, 67), and antiarrhythmic drug infusion (12, 49). In the present study, both epicardial and endocardial strength-interval curves have been found to exhibit a smooth hyperbolic shape with no dips on the steep descending portion of the curve (Fig. 2), suggesting that no supernormal excitability phase, an attribute of anodal break-excitation (20, 33, 45), was yielded by bipolar stimulations.

Previous studies have demonstrated close correlation between diastolic excitability threshold and VF threshold values (15, 29, 33, 36, 62), suggesting that increased tissue excitability may facilitate VF induction. The LV was found to be most susceptible to electrically induced VF at those intervals of the cardiac cycle at which the ventricular capture threshold is the lowest (15, 33, 36). Furthermore, there is evidence to suggest that factors [e.g., ischemia, hyperkalemia, or infusion of antidysrhythmic agent (class 1b antiarrhythmic agent)] that influence excitability threshold in canine ventricular muscle may cause similar changes in VF threshold (29, 62). In this regard, lowered ventricular late diastolic thresholds have been associated with enhanced vulnerability to arrhythmias induced by coronary artery ligations in anesthetized dogs (45). In the present study, we found that greater endocardial Na⁺ protein expression levels are associated with greater excitability at endocardial than epicardial stimulation sites. Indeed, the ventricular excitation thresholds as measured during both regular pacing and premature extrasystolic stimulation at progressively decreasing S1–S2 intervals were found to be lower at endocardium than epicardium (Fig. 2). These changes were associated with lower VF thresholds, and greater susceptibility to stimulation-evoked tachyarrhythmias, at endocardium than epicardium.

Importantly, modeling studies suggest that in the presence of higher tissue excitability an extrasystolic pulse of a given strength may activate more ventricular cells, produce greater voltage gradients, and create a larger area of slowly propagating graded responses, thereby providing a favorable electrophysiological substrate to initiate reentry (25). In a model of a ring-shaped, one-dimensional cardiac fiber, the uniform reductions in excitability produced by lowering the Na⁺ channel conductance have been associated with decreased inducibility...
of unidirectional conduction block and reentry (58). These changes are accounted for by the reductions in size of the vulnerable window at the end of repolarization, over which a premature extrastimulus may initiate reentry, and by the shift of the vulnerable window toward a more repolarized portion of the action potential, where the spatial voltage gradients are reduced (58). In cardiac Purkinje fibers, enhanced excitability has been shown to promote conversion of the stable stimulus-response pattern into highly irregular activity with APD alternans (16). Importantly, experimental and modeling studies suggest that enhanced excitability contributes to a reduced central core area around which the spiral wave rotates during VF (47, 54). This change contributes to maintenance of sustained VF because of a reduced rotation period of the spiral wave and therefore increased dominant frequency of ventricular activation (47, 54). Taken together, these mechanisms may account for the association of greater excitability and increased susceptibility to electrical stimulation-evoked tachyarrhythmias at ventricular endocardium than epicardium, as observed in the present study.

**ERP restitution and susceptibility to VF: the mechanisms.**

Electrical restitution refers to rate-dependent adaptation of ventricular action potential duration (10). Analysis of electrical restitution by plotting APD90 or ERP as a function of the preceding DI has been used widely to predict propensity to VF in experimental and clinical studies (13, 51, 68, 83). A strong negative correlation has been found between the maximal restitution slope values and VF threshold values assessed during vagosympathetic stimulations in perfused rabbit heart preparations (50).

Steep electrical restitution promotes VF by inducing the repolarization alternans that is manifested as regular beat-to-beat oscillations in APD60 (Fig. 4). Once the heart is paced at a constant cycle length, a sudden shortening of action potential in one beat contributes to prolongation of the following DI, and therefore increased APD60, in the next beat. This in turn is followed by reduced DI and hence shortened action potential, thereby initiating repolarization alternans. In the presence of steep electrical restitution, the magnitude of repolarization alternans is incrementally amplified over successive cardiac cycles, an effect ultimately leading to local conduction block that precipitates VF due to breakup and multiple fragmentation of the excitation wave front (13, 83). In contrast, in the presence of flat electrical restitution, the magnitude of repolarization alternans never reaches the critical value at which VF is induced, because initial APD change is gradually dampened over the next cardiac cycles (13, 83).

Alternatively, repolarization alternans may be attributed to restitution-independent mechanisms such as abnormal Ca2+ handling at fast beating rates. In support of this notion, repolarization alternans is suppressed by agents that eliminate Ca2+ entry into the cell, deplete sarcoplasmic reticulum Ca2+ stores, or buffer intracellular free Ca2+ (30, 35, 80). In dog endocardial tissue, beat-to-beat oscillations in APD60 may be elicited by a pacing protocol that preserves a constant value of DI (85). Nevertheless, the amplitude of repolarization alternans was found to be lower after “constant DI” pacing than during a “constant cycle length” stimulation protocol that allows DI changes, thus highlighting the importance of restitution-dependent mechanisms (85).

**Stimulation protocols used.** The electrical restitution may be assessed with either a dynamic stimulation protocol in which the heart is paced at progressively increasing regular rates or a standard stimulation protocol utilizing extrasystolic stimulation at variable S1-S2 intervals. Studies on canine heart preparations suggest that the dynamic restitution protocol is superior to the standard protocol at predicting enhanced susceptibility to VF (41, 61), thereby highlighting the importance of using rapid cardiac pacing to compare arrhythmogenicity at epicardium and endocardium in the present study. Rapid cardiac pacing is also a more aggressive stimulation protocol than extrasystolic stimulations, and therefore it may be optional to detect even moderate changes in arrhythmic susceptibility with a limited sample size. On the other hand, the extrasystolic stimulation protocol in which the premature extrastimulus is interpolated in between two regular pulses may be more relevant to study tachyarrhythmias resulting from the interference of intrinsic escaped beats with the regular pacemaker rhythm in pacemaker-implanted cardiac patients (86). Therefore, both tachypacing andextrasystolic stimulation protocols were used in our study.

**Epicardial-to-endocardial difference in ERP restitution and arrhythmic susceptibility.** In the present study, we found that endocardial stimulation sites exhibit steeper ERP restitution slopes and faster restitution kinetics than epicardial stimulation sites (Fig. 3), changes associated with higher magnitude of repolarization alternans, and greater VT inducibility upon endocardial tachypacing (Fig. 4). In line with these findings, Wan et al. (80) previously showed greater susceptibility to repolarization alternans in endocardial than epicardial myocytes dissociated from guinea pig ventricular tissue.

We sought to determine whether our findings may be ascribed to nonuniform distribution of Na+ channels across ventricular wall. Indeed, greater endocardial Na+ protein expression levels may imply greater availability of Na+ channels for activation over the late phase of ventricular repolarization, thereby contributing to epicardial-to-endocardial difference in ERP restitution. In support of this argument, tetrodotoxin-induced Na+ channel block has been associated with greater shortening of action potential in subendocardial than subepicardial guinea pig myocytes (46). Importantly, we found that the epicardial-to-endocardial difference in electrical restitution kinetics was eliminated by flecainide, a Na+ channel blocker, which reduced the steepness of ERP restitution and slowed its time course (Fig. 6 and Table 2). These changes are consistent with computer simulations demonstrating that eliminating the rate-dependent changes of INa in a model of guinea pig ventricular myocyte reduces the maximal slope of electrical restitution over a range of short DIs (57). Taken together, our results support the notion that greater Na+ channel availability contributes to steeper electrical restitution slopes, thus providing a mechanism for epicardial-to-endocardial difference in ERP restitution kinetics, as well as differential susceptibility to repolarization alternans and tachyarrhythmia following rapid cardiac pacing.

**Spatial repolarization gradients while pacing at different ventricular stimulation sites.** In line with previous studies (51, 52, 55), we found that epicardial APD90 values are greater in the RV than the LV chamber in the guinea pig heart (Fig. 5, A and B). This difference is thought to be ascribed to asymmetric distribution of IK1, the inward rectifier K+ current, with greater Kir2.1 and Kir2.3 expression levels in the LV than the RV.
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chamber (65, 78, 81). Regarding transmural dispersion of repolarization, although studies on isolated guinea pig ventricular cells revealed greater APD₉₀ values in endocardial than epicardial myocytes due to greater epicardial delayed rectifier current [rapid (Iₖ,δ) and slow (Iₖ,α) components] density (11, 46), no consistent evidence in support of these relationships has been found in studies on perfused ventricular tissue. In whole heart preparations or isolated strips of ventricular muscle, endocardial APD₉₀ was reported to be shorter than (39), slightly (a few milliseconds) longer than (82), or similar to (52, 71) epicardial APD₉₀ in the guinea pig. In the present study, we found a small positive difference (5–10 ms) to exist between endocardial and epicardial APD₉₀ values determined at the LV anterior wall (Fig. 5, D–F).

Importantly, differential arrhythmic susceptibility to epicardial and endocardial pacing was not related to changes in spatial repolarization gradients (Fig. 5, C and F). In this regard, our findings are at odds with studies on canine and rabbit ventricular wedge preparations showing longer QT interval and greater transmural dispersion of repolarization during epicardial than endocardial pacing (24, 48). In canine ventricular wedges, these changes may translate to greater epicardial than endocardial VT inducibility by programmed stimulation, at least in the presence of drug-induced long-QT syndrome (24, 76). Presumably, both species-related changes and methodological differences (e.g., using whole perfused hearts rather than ventricular wedge preparations) may account for these discrepancies. Of note, no differences in Na⁺ channel mRNA expression levels, Jᵥₚ, density, or ventricular pacing thresholds have been found between epicardial and endocardial layers in canine hearts (6, 18, 63). These findings may imply the limited value of the canine model for studies on the role of nonuniform Na⁺ channel distribution in epicardial-to-endocardial heterogeneities, as observed in human hearts.

No consistent evidence in support of differential effects of epicardial versus endocardial stimulation on duration of ventricular repolarization or spatial repolarization gradients has been obtained in human studies. Although some studies showed increased transmural dispersion of repolarization during LV epicardial pacing (5, 48), others reported reduced rather than increased QT interval and T peak-to-end interval values, as well as QT interval dispersion, while switching from RV endocardial to biventricular (RV endocardial plus LV epicardial) pacing (7, 66, 77).

Limitations. In the present study, we did not perform patch-clamp recordings in cardiac myocytes dissociated from different ventricular layers to validate transmural gradient in distribution of Na⁺ channels. However, in previous studies, both the amplitude of phase 0 and the maximal velocity of action potential upstroke (Vₘₐₓ) were found to be higher in endocardial than epicardial myocytes from the guinea pig heart (11, 71, 79, 82), findings suggesting greater availability of Na⁺ channels at ventricular endocardium.

There is a possibility that endocardial pacing is associated with direct stimulation of Purkinje fibers, which may contribute to greater propensity for tachyarrhythmias during endocardial than epicardial stimulation. However, similar values of QRS complex duration have been found after epicardial and endocardial stimulations applied over a wide range of pacing intervals (Fig. 7), thus suggesting that the fast-conducting Purkinje system is unlikely to govern ventricular depolarization during endocardial pacing. In this regard, there is evidence to demonstrate that activation of the ventricular free wall after endocardial pacing is mostly determined by myocardial wave fronts rather than the peripheral conduction system (14). Mapping of endocardial activation sequence in isolated, perfused rabbit heart preparations has revealed that although in sinus rhythm Purkinje fiber depolarization always precedes myocardial wave fronts, during endocardial pacing peripheral conduction system wave fronts lagged behind myocardial depolarization in majority of recording sites (14). Importantly, subendocardial Purkinje fibers in the guinea pig heart are surrounded by connective tissue sheaths that separate them from adjacent contractile muscle (1, 21), and presumably may insulate against the direct effect of the stimulating current produced by the endocardial pacing electrode.

Local conduction heterogeneities are known to promote reentrant tachyarrhythmia (19). However, in the present study, we did not examine whether the endocardial-to-epicardial difference in excitability may translate into appropriate changes in conduction velocity and, if so, whether these changes may contribute to differential arrhythmic susceptibility. Importantly, even with direct measurements of conduction velocity, it would be difficult to prove that endocardial tachypacing is associated with no local conduction abnormalities in epicardial regions with low excitability. Computational studies using the guinea pig myocyte model demonstrate that the safety factor of ventricular conduction is relatively insensitive to moderate changes (e.g., up to 50% reduction in Na⁺ channel conductance) in membrane excitability (69). Of note, Scn5a heterozygous mice (which have ~40% reduction in peak Jᵥₚ), although found to show a slowed conduction velocity in the RV chamber, nevertheless do not exhibit any change in susceptibility to electrically induced tachyarrhythmias compared with wild-type mice (73). Although these findings suggest that differential Na⁺ channel expression levels at ventricular epicardium and endocardium are unlikely to cause proarrhythmic conduction abnormalities in our experiments, further studies utilizing computer models of ventricular activation are warranted to characterize the wave front propagation upon tachypacing and extrasystolic stimulation in the presence of epicardial-to-endocardial difference in excitability.

Importantly, class 1c agents were found to produce proarrhythmic effects in cardiac patients with recent myocardial infarction (75), which may be ascribed to markedly depressed conduction in the ischemic zone in the presence of relatively preserved conduction in normal ventricular tissue, changes establishing the electrophysiological substrate for reentrant tachyarrhythmia (60). These conduction abnormalities may be attributed to nonuniform changes in excitability produced by Na⁺ channel blockers in infarcted hearts. Indeed, a nonhomogeneous excitability (e.g., where a depressed region is interposed between segments with normal excitability) was shown to cause discontinuities in conduction and set a stage for reflected reentry (64). In this regard, our findings on differential arrhythmic susceptibility to epicardial and endocardial pacing apply only to normal hearts, showing no local inhomogeneities in excitability at either epicardium or endocardium.

The present study was designed to explore the distribution pattern of Na⁺ channels across ventricular wall and to determine its functional correlates. As such, it may add to better understanding of the mechanisms contributing to arrhythmogenicity in patients subjected to endocardial stimulation (9,
70). Our findings, however, do not necessarily imply the superior value of ventricular epicardial versus endocardial pacing. Indeed, epicardial pacing, although widely used in pediatric cardiology (17), has well-recognized intrinsic limitations. Greater epicardial than endocardial pacing thresholds may contribute to as much as threefold higher energy consumption during epicardial pacing, thereby reducing the lifetime of the epicardial pacemaker (23). In heart failure patients, LV epicardial pacing is associated with less hemodynamic improvement than LV endocardial pacing (28), which may be partly ascribed to a reversed sequence of transmural LV activation.

Importantly, greater epicardial Na\(^+\) channel expression levels and resultant changes in arrhythmic susceptibility may not fully account for the whole range of detrimental effects associated with RV endocardial pacing, including asynchronous ventricular activation, abnormal myocardial perfusion, and changes in ventricular innervation pattern (74). Finally, the present findings rely on the use of the isolated, perfused guinea pig heart model free of any structural changes caused by cardiac disease. Further studies are warranted to determine whether these findings may be used to explain the epicardial-to-endocardial heterogeneities existing in the heart of large mammalian species including humans, or may contribute to arrhythmogenic mechanisms operating in failing hearts when assessed in vivo.

Concluding remarks. In summary, the present study demonstrates that higher Na\(^+\) channel expression levels may account for greater excitability, steeper ERP restitution slopes and faster restitution kinetics, shorter minimal pacing intervals that allow 1:1 ventricular capture, higher susceptibility to repolarization alternans, and greater propensity to electrical stimulation-induced tachyarrhythmias at ventricular endocardium than epicardium in the guinea pig heart.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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