Larger late sodium conductance in M cells contributes to electrical heterogeneity in canine ventricle

ANDREW C. ZYGMUNT, GEOFFREY T. EDDLESTONE, GEORGE P. THOMAS, VLADISLAV V. NESTERENKO, AND CHARLES ANTZELEVITCH

Department of Experimental Cardiology, Masonic Medical Research Laboratory, Utica, New York 13501-1787

Received 5 December 2000; accepted in final form 24 April 2001

Zygmont, Andrew C., Geoffrey T. Eddlestone, George P. Thomas, Vladislav V. Nesterenko, and Charles Antzelevitch. Larger late sodium conductance in M cells contributes to electrical heterogeneity in canine ventricle. Am J Physiol Heart Circ Physiol 281: H689–H697, 2001.—Action potentials and whole cell sodium current were recorded in canine epicardial, midmyocardial, and endocardial myocytes in normal sodium at 37°C. Tetrodotoxin (TTX) reduced the action potential duration of midmyocardial cells to a greater degree than either epicardial or endocardial cells. Whole cell recordings in potassium-free and very-low-chloride solutions revealed a slowly decaying current that was completely inhibited by 5 μM TTX or replacement of external and internal sodium with the impermeant cation N-methyl-D-glucamine. Late sodium current density at 0 mV was 47% greater in midmyocardial cells and averaged −0.532 ± 0.058 pA/pF in endocardial, −0.463 ± 0.068 pA/pF in epicardial, and −0.785 ± 0.070 pA/pF in midmyocardial cells. Neither the frequency dependence of late sodium current nor its recovery from inactivation exhibited transmural differences. After a 4.5-s pulse to −30 mV, late sodium current recovered with a single time constant of 140 ms. We conclude that a larger late sodium conductance in midmyocardial cells will favor longer action potentials in these cells. More importantly, drugs that slow inactivation of sodium channels will produce a nonuniform response across the ventricular wall that is proarrhythmic.

ACTION POTENTIALS (AP) across the canine left ventricular free wall exhibit distinctly different morphologies, responses to pharmacological agents, and rate dependence. Unequal distribution of ion channels contributes to this transmural electrical heterogeneity and thus to the development of a variety of cardiac arrhythmias (2). Both epicardial (Epi) and midmyocardial (M) tissues and isolated cells display a spike and dome morphology that is not observed in the endocardium. The absence of an endocardial (Endo) notch has been correlated with a smaller 4-aminopyridine-sensitive potassium conductance [transient outward K⁺ current (Iₒₒ₅)] in the canine endocardium (17, 19). Similarly, a greater prolongation of the M cell AP in response to either a reduced pacing rate or inhibition of delayed rectifier potassium channels has been associated with transmural differences in a repolarizing current. Compared with Epi and Endo cells, the slowly activating component of the delayed rectifier potassium conductance (Iₖₛ) is smaller in M cells (18). The significance of a smaller midmyocardial Iₖₛ can only be judged when the transmural distribution of opposing currents active during the cardiac plateau is known.

The observation that low concentrations of lidocaine that have little effect on maximal upstroke velocity (Vₒₒ₅) can markedly reduce the AP duration at 90% repolarization (APD₉₀) of canine ventricular myocytes indicates the importance of a slowly inactivating or late sodium conductance in the maintenance of the action potential plateau (30). Characterization of this conductance in cells from the three different layers of the ventricular free wall is the focus of this study. There is evidence (3) that sodium current (Iₙₐ) density may not be uniform across the ventricular wall because Vₒₒ₅ measured in isolated tissues from the midmyocardium is significantly greater than that in either the endocardium or epicardium. If the same channel population underlies both the early and late sodium conductance, this suggests that there are differences in the density of this plateau-sustaining conductance across the ventricular wall.

We examined the contribution of late sodium conductance to AP morphology and quantified this conductance in the three regions of the ventricular wall. Our data confirm that Iₙₐ sustains the plateau of the AP and that the density of this conductance is significantly higher in the midmyocardium than in other regions of the ventricular wall. Aspects of this work have been published as an abstract (10).

METHODS

Adult male mongrel dogs were given heparin sodium (200 IU/kg) and anesthetized with pentobarbital sodium (35 mg/kg iv), and their hearts were quickly removed and placed in Tyrode solution. Single myocytes were obtained by enzy-
mantic dissociation from a wedge-shaped section of the ventricular free wall supplied by the left circumflex coronary artery (34). Cells from the Epi, M, and Endo regions of the left ventricle were used in this study. All of the procedures followed were in accordance with guidelines established by the Institutional Animal Care and Use Committee.

Tyrode solution used in the dissociation contained (in mM) 135 NaCl, 5.4 KCl, 1 MgCl₂, 5 NaH₂PO₄, and 10 HEPES, and pH was adjusted to 7.4 with NaOH. A standard patch-clamp technique was used to record whole cell currents at 37°C. The composition of the external solution was (in mM) 2 CaCl₂, 1 MgCl₂, 10 glucose, 140 sodium-methane sulfonate, and 10 HEPES, and pH was adjusted to 7.4 with methane sulfonic acid. Pipette solution contained (in mM) 155 NaCl, 5.4 KCl, 1 MgCl₂, 0 or 0.5 CaCl₂, 10 glucose, 0.33 CsOH. When it was necessary to abolish all movement of monovalent ions through sodium channels, the external solution contained (in mM) 2 CaCl₂, 1 MgCl₂, 10 glucose, 140 N-methyl-D-glucamine methane sulfonate, and 10 HEPES, and pH was adjusted to 7.4 with methane sulfonic acid. Pipette solution contained (in mM) 155 N-methyl-D-glucamine methanesulfonate, 10 HEPES, 1 MgCl₂, 5 MgATP, and 2 EGTA, and pH was adjusted to 7.1 with CsOH. When it was necessary to abolish all movement of monovalent ions through sodium channels, the external solution contained (in mM) 2 CaCl₂, 1 MgCl₂, 10 glucose, 140 sodium-methane sulfonate, and 10 HEPES, and pH was adjusted to 7.4 with NaOH. Pipette solution contained (in mM) 0.00026 amphotericin B, 135 potassium aspartate, 10 NaCl, 10 KCl, 10 HEPES, and pH was adjusted to 7.1 with KOH. We have assumed that intracellular potassium, sodium, and chloride equilibrate with pipette solution within minutes of incorporation of amphotericin B in the membrane, at rates similar to those suggested for the nystatin perforated patch technique (14).

Amphotericin B (Sigma) was made in dimethyl sulfoxide (60 mg/ml) and diluted 1:250 into pipette solution to a final concentration of 240 μg/ml. Tetrodotoxin (TTX) was made in water and diluted to final concentrations between 0.3 and 15 μM in external solution. Amphotericin B was used in a darkened room.

Dissociated cells were placed in a temperature-controlled 0.5-ml chamber (Medical Systems; Greenvale, NY) on the stage of an inverted microscope and superfused at 2 ml/min. A four-barrel quartz micromanifold (ALA Scientific Instruments; Westbury, NY) was used to apply TTX. This micromanifold was placed 100 μm from the cell, and flow was controlled by a pinch valve and computer interface (model BPS-4, ALA Scientific Instruments). An Axopatch 200A amplifier (Axon Instruments; Foster City, CA) was operated in current- or voltage-clamp mode to record, AP or ionic currents, respectively, at 37°C. Cell capacitance was 203 ± 3 pF (64 cells). Pipette tip resistance was 1.0–1.5 MΩ when whole cell currents were recorded and 5–10 MΩ when AP were recorded. Seal resistance was >5 GΩ. Electronic compensation of series resistance averaged 76 ± 2%, and the series resistance remaining after this compensation averaged 0.963 ± 0.061 MΩ. After series resistance compensation, capacitive current decayed with a single time constant of 206 ± 12 ms.

Voltages reported in the text were corrected for patch electrode tip potentials (35). Tip potentials for perforated patch solution averaged 15 ± 0.25 mV (32 cells). Tip potentials were 11 ± 0.93 mV (5 cells) and 13 ± 0.99 mV (5 cells) when patch electrodes were filled with cesium or N-methyl-D-glucamine, respectively. The seal between cell membrane and patch pipette was initially formed in Tyrode solution containing 1 mM of CaCl₂. A 3 M KCl-agar bridge was used between the Ag/AgCl ground electrode and external solution to avoid development of a ground potential when switching to experimental solution.

AP were recorded at a basic cycle length of 15 s. The APD₉₀ was computed as the time between the triggering stimulus and repolarization of transmembrane potential to 12 mV positive of the resting potential.

Late Iᵥ Na density, frequency dependence, and reactivation kinetics were measured in the three cell types. Frequency dependence and reactivation kinetics were ultimately presented as pooled data because of a lack of differences across the ventricular wall in these measurements. Late Iᵥ Na density was recorded in cells that were held at −80 mV. To remove steady-state inactivation, a 2,000-ms pulse to −130 mV was taken before a 500-ms pulse to voltages between −40 and 0 mV, and the entire protocol was repeated at intervals of 30 s. Tyrode solution at a concentration of 10 μM was applied and these voltage steps were repeated. Late Iᵥ Na characterized as the TTX-sensitive difference current, was measured as the average current 30–35 ms and 295–300 ms after the beginning of the test pulse. Measurements made in the three cell types were normalized by dividing these currents by cell capacitance and plotted as a function of voltage.

After a 30-s rest at the holding potential, the frequency dependence of late Iᵥ Na was evaluated during a train of 400-ms pulses to −20 mV that were repeated every 500 ms. Iᵥ Na was characterized as the TTX-sensitive difference current measured 30–35 and 395–400 ms after the beginning of the test pulse. Averaged data for the three cell types were normalized to the fully activated late Iᵥ Na and plotted as a function of pulse number.

Reactivation of late Iᵥ Na was determined using a dual-pulse protocol in which two 4,500-ms pulses to −30 mV were separated by a variable interpulse interval. Both holding and interpulse potentials were −80 mV and interpulse intervals were varied from 5 to 400 ms. Current traces during pulse 1 and pulse 2 were fitted with a double-exponential function of the form

\[ I(t) = A_{\text{fast}} \exp(-t/\tau_{\text{fast}}) + A_{\text{slow}} \exp(-t/\tau_{\text{slow}}) + C \]

where \( I \) is current, \( A_{\text{fast}} \) is the amplitude of the fast component, \( A_{\text{slow}} \) is the amplitude of the slow component, \( \tau_{\text{fast}} \) is the time constant of the fast component, \( \tau_{\text{slow}} \) is time constant of the slow component, and \( t \) is time. For interpulse intervals ≥50 ms, double-exponential fits accurately followed the time course of current decay, whereas single-exponential fits failed to describe inactivation with a reasonable accuracy. However, after interpulse intervals <50 ms, Iᵥ Na decay was monoexponential with only the slow component remaining.

Reactivation was analyzed quantitatively by using these exponential fits. For each interpulse interval, we separately estimated time-dependent (\( A_{\text{fast}} + A_{\text{slow}} \)) and time-independent components (\( C \)) of late Iᵥ Na by calculating corresponding average values for both pulse 1 (\( A_{\text{fast}} + A_{\text{slow}}, C_1 \)) and pulse 2 (\( A_{\text{fast}} + A_{\text{slow}}, C_2 \)). Results were expressed as

\[ \frac{A_{\text{fast}} + A_{\text{slow}}}{A_{\text{fast}} + A_{\text{slow}} + C_2} \] and plotted as a function of interpulse interval.

Whole cell currents and transmembrane potentials were filtered with a four-pole low-pass Bessel filter at 5 kHz.
digitized between 2 and 5 kHz (Digidata 1200A, Axon Instruments), and stored on a computer. Traces were additionally digitally filtered with a Gaussian filter at 500 Hz (Clampfit 8, Axon Instruments). Significant differences between means were determined by an unpaired Student's t-test or two factor with replication analysis of variance. Data acquisition and analysis software (Clampex 8, Axon Instruments) was used to record transmembrane potential or ionic current.

RESULTS

Figure 1 demonstrates the effect of low concentrations of TTX on AP in cells isolated from Endo and M regions of the ventricular wall. The basic cycle length of 15,000 ms was used to emphasize the prolongation of the M cell AP at slow rates. Data were gathered for controls and then following application of TTX in the range of 0.1 to 10 μM. In some experiments, TTX was incremented stepwise; in others, TTX was washed out before changing the concentration to assure reversibility and to control for possible time-dependent changes in AP morphology. Figure 1A shows that the first consistent effect on AP morphology was observed at 0.3 μM TTX, which resulted in a TTX-induced acceleration of phase 3 repolarization and attendant reduction of APD90 in Endo and M cells. Within the endocardium, AP shortening was maximal at 3 μM TTX. In Epi and M cells, concentrations of TTX >0.3 μM caused progressive deepening of the AP notch. Depending on the initial depth of the notch, increasing TTX to 3 or 10 μM caused either a pronounced delay in the upstroke of phase 2 (see Fig. 1A, top) or complete loss of the dome in both Epi and M cells. The effects of TTX shown in Fig. 1A are representative of recordings in eight Endo and nine M cells.

To compare TTX-induced changes in APD90, we selected a concentration of TTX that reduced APD90 without altering depth of the notch, because a hyperpolarized notch will secondarily modify development of other conductances (37). Figure 1B illustrates that the absolute reduction of APD90 achieved with 0.3 μM of TTX was not uniform across the ventricular wall. Reduction was greatest in M cells because the APD90 here is considerably greater than that of either Epi or Endo cells at a basic cycle length of 15,000 ms. Responses from representative cells for a control AP and one recorded in the presence of 0.3 μM TTX are shown for the three cell types. In these examples, TTX reduced APD90 by 36, 209, and 28 ms in Epi, M, and Endo cells, respectively. Similar results were observed in five experiments on each cell type.

These results indicate that late sodium conductance contributes to maintaining the AP plateau in the different cell types across the wall of the canine left ventricle. To determine whether the density of this conductance is larger in M cells and might therefore be a factor in M cell AP prolongation at slow pacing rates, I$_{Na}$ was measured directly by using whole cell voltage clamp technique. Late I$_{Na}$ was measured using a potassium-free and very-low-chloride medium containing 140 mM sodium in the experimental chamber with a pipette solution containing 10 mM sodium. Protocols were initiated 4 min after membrane rupture. A 2,000-ms pulse to −130 mV preceded each measure-
Figure 2 illustrates that the density of late \( I_{\text{Na}} \) is greatest in M cells. In Fig. 2A, currents measured at 0 mV in an M cell are shown before TTX (largest inward trace) and after 10 \( \mu \text{M} \) TTX has completely blocked late \( I_{\text{Na}} \). Subtraction of the TTX trace from the control trace generated the TTX-sensitive difference current shown in Fig. 2B. Similar subtractions were used to determine late \( I_{\text{Na}} \) in Epi and Endo cells over a range of potentials, and these data are summarized in Fig. 2, C and D. The mean TTX-sensitive current during intervals of 30–35 ms and 295–300 ms after the start of the depolarizing pulse are shown in Fig. 2, C and D, respectively. These plots clearly indicate that a greater density of late \( I_{\text{Na}} \) is encountered in the midmyocardium over a wide range of voltages. After 295 ms at 0 mV, mean \( I_{\text{Na}} \) was \(-0.532 \pm 0.058 \) pA/pF (12 cells) in Endo cells, \(-0.463 \pm 0.068 \) pA/pF (13 cells) in Epi cells, and \(-0.785 \pm 0.070 \) pA/pF in M cells (13 cells, \( P < 0.001 \)). These transmural differences of an inward current active at plateau potentials will favor a longer AP in M cells at slow rates.

To verify that the TTX-sensitive difference current truly represents \( I_{\text{Na}} \), monovalent cations on both sides of the membrane were substituted by the impermeant cation N-methyl-D-glucamine. In 12 cells, TTX-sensitive currents could not be detected during 500-ms voltage-clamp steps to \(-40, -20, 0 \) mV under these conditions. Measurements of late \( I_{\text{Na}} \) were conducted in normal external sodium at 37°C. It is well known that activation of sodium channels under these conditions results in transient loss of voltage control (35). If fast \( I_{\text{Na}} \) is abolished or significantly reduced by 10 \( \mu \text{M} \) TTX, then the voltage overshoot that occurs when stepping to the test potential will not be the same after TTX. The TTX-sensitive current could then be contaminated by calcium current (\( I_{\text{Ca}} \)). To assess the degree to which these perturbations of the voltage profile affect our results, we first examined the effect of 10 \( \mu \text{M} \) TTX on fast \( I_{\text{Na}} \). \( I_{\text{Na}} \) was recorded in cells that were held at \(-80 \) mV. To remove steady-state inactivation, a 2,000-ms pulse to \(-130 \) mV was taken before a 500-ms pulse to \(-30 \) mV. In Fig. 3A, \( I_{\text{Na}} \) was so large as to be clipped by the headstage. This large amplitude current should cause a transient loss of voltage control lasting 2.5–3 ms (35). TTX was applied and the protocol repeated. In Fig. 3B, TTX speeded the rate of decay of this current, but the amplitude was still sufficient to be clipped by the headstage and should have also resulted in a transient voltage overshoot. Because the decay of \( I_{\text{Na}} \) was altered by TTX, we expect that this voltage overshoot will be slightly different in its decay. The decay of \( I_{\text{Na}} \) during the first 5 ms of the step was fit to a single exponential. This time constant was \(0.41 \pm 0.021 \) ms in control and \(0.23 \pm 0.018 \) ms after TTX (\( P < 0.001; n = 5 \) cells).

These results suggest that pulses between \(-40 \) and 0 mV will temporarily exceed these voltages before decaying to the test potential. \( I_{\text{Ca}} \) will be activated during this overshoot, and the degree of activation will be altered by TTX, causing \( I_{\text{Ca}} \) to become a component of the TTX-sensitive current. This TTX-induced contribution of \( I_{\text{Ca}} \) is itself transient and does not extend throughout the entire 500-ms test pulse. Moreover, \( I_{\text{Ca}} \) will contribute for a shorter period of time at a test potential of \(-40 \) mV than at 0 mV because calcium channels activated during the overshoot close rapidly at \(-40 \) mV.

We then tested the effects of dramatically altering the overshoot that normally occurs at the beginning of each test pulse. Although these changes in voltage...
profile will not be equivalent to that expected after applying TTX, this approach permits some measure of the changes in $I_{Ca}$ caused by alteration of the overshoot. We have previously reported that stepping to 0 mV from a holding potential of $-80$ mV resulted in an overshoot lasting $<3$ ms. Introducing a 5-ms prestep to $-50$ mV caused loss of voltage control during this prestep but not during the subsequent step to 0 mV (35). We used this same approach to examine two independent cases in the present study, one in which a step from the holding potential resulted in an overshoot at the beginning of the test step and a second in which a prepulse to $-50$ mV eliminated this overshoot during the subsequent test step. Figure 3 shows the effect of a 5-ms prepulse to $-50$ mV on currents evoked by a 500-ms test pulse to $-40$ mV (Fig. 3C) or 0 mV (Fig. 3D). We picked these two voltages because the behavior of $I_{Ca}$ is very different at $-40$ and 0 mV. In Fig. 3, C and D, we show superimposed traces with and without a prepulse. The dotted lines are placed 30 and 295 ms after the start of the test pulse, at times used to measure late $I_{Na}$. Although it is not obvious because the two traces are superimposed, the prepulse eliminated the inward spike of fast $I_{Na}$ at the very beginning of each test step and caused a decrease in the earliest phase of $I_{Ca}$ at 0 mV. Whereas the prepulse altered currents at the beginning of the test step, it did not affect current 30 or 295 ms after the start of the test pulse at either of these voltages. Similar results were obtained in six additional cells.

These prepulse experiments must be viewed carefully because they do not exactly duplicate the TTX-induced alterations in the time course of the fast $I_{Na}$ that presumably bring about changes in the voltage overshoot and activation of $I_{Ca}$.

A larger late $I_{Na}$ in M cells does not result from a differential sensitivity of the three cell types to TTX. Figure 4 shows the complete inhibition of late $I_{Na}$ by 5 μM TTX and the failure of 10 and 15 μM TTX to block.
additional currents in Epi (Fig. 4A) and M cells (Fig. 4B) during 400-ms steps to −40 mV. TTX at a concentration of 5 μM completely inhibited late \( I_{\text{Na}} \) measured 30 and 295 after the beginning of a pulse to −40 mV in 5 cells from each of the three ventricular layers. In these 15 cells, we found no variation in TTX-sensitive currents when comparing difference currents produced by 5, 10, and 15 μM TTX at voltages of −40, −20, and 0 mV. We conclude that the data summarized in Fig. 2, C and D, indicate a higher density of late \( I_{\text{Na}} \) in M cells rather than a failure of 10 μM TTX to block all late \( I_{\text{Na}} \) in Epi and Endo cells.

The transmural gradient summarized in Fig. 2, C and D, was measured after a pause of 30 s. If reactivation of late \( I_{\text{Na}} \) is slower in M cells than in Epi and Endo cells, rapid pacing will reduce \( I_{\text{Na}} \) in M cells to a greater degree and diminish differences in both late \( I_{\text{Na}} \) and APD90 across the wall. Frequency dependence of the TTX-sensitive current was measured in 11 cells from each of the 3 layers, and two-factor analysis of variance was applied to the results to determine significant differences among the layers. Cells were rested for 30 s at the holding potential before evoking a train of 30 pulses of 400 ms duration, which were repeated every 500 ms. \( I_{\text{Na}} \) was normalized by dividing all traces by the current during pulse 1. Mean \( I_{\text{Na}} \) measured 30 and 395 ms after the beginning of each pulse is shown in Fig. 5, A and B, respectively. The percent reduction of \( I_{\text{Na}} \) throughout the train was similar across the ventricular wall. Results from the three cell types were pooled (33 cells). Comparing currents during the first and last pulses, \( I_{\text{Na}} \) at 30 ms was reduced by 32%, whereas current at 395 ms was reduced 25%.

The rate of recovery from inactivation was investigated by means of a dual-pulse protocol, in which two 4.5-s pulses to −30 mV were separated by a variable interpulse interval. The decay and reactivation of \( I_{\text{Na}} \) was similar in the three cell types and results were pooled in Fig. 6. Reactivation was quantified by fitting exponentials to the decay of \( I_{\text{Na}} \) during pulse 1 and by measuring the restoration of time-dependent and time-independent components during pulse 2. For interpulse intervals 50 ms, time constants for the decay of the current during pulse 2 were nearly identical to those in pulse 1. Fast time constants were 108 ± 6 ms (mean ± SE; \( n = 63 \)) and 107 ± 10 ms (mean ± SE; \( n = 47 \)) for pulse 1 and pulse 2, respectively. Pulse 1 and pulse 2 slow time constants were 2,000 ± 860 and 1,930 ± 1,000 ms. Shown in Fig. 6 is the recovery of time-dependent (Fig. 6A) and time-independent components (Fig. 6B) as a function of interpulse interval. A single exponential with a time constant of 140 ms described the restoration of time-dependent current at −80 mV. Figure 6B indicates that the steady-state component of late \( I_{\text{Na}} \) recovered instantaneously to the current level at the end of pulse 1. Relative amplitudes of the fast, slow, and instantaneous late \( I_{\text{Na}} \) currents during the initial pulse were 0.58 ± 0.04, 0.19 ± 0.02, and 0.23 ± 0.02 (mean ± SE; \( n = 63 \)).

**DISCUSSION**

The focus of our study was to delineate transmural differences of late \( I_{\text{Na}} \) in a bid to understand the distinctive response of M cells to agents that selectively affect sodium channels. We have shown that a larger late \( I_{\text{Na}} \) in the canine midmyocardium contributes to the longer AP of M cells. A higher density of sodium channels, a larger single-channel conductance, or an increased probability of channel reopening must underlie this larger whole cell current. Wasserstrom and Salata (30) uncovered the contribution of late \( I_{\text{Na}} \) to the cardiac plateau in the canine ventricle, but they did not distinguish among Epi, M, and Endo myocytes. A larger late \( I_{\text{Na}} \) is not the only basis for prolonged M cell APs, because a weaker \( I_{\text{Ks}} \) and stronger electrogenic \( I_{\text{NaCa}} \) also contribute (18, 36). A persistent TTX-sensitive conductance has also been reported (4, 5, 11, 15) in cardiac pacemaker cells from toads and Purkinje fibers from dogs, sheep, and rabbits. Opposite to our findings, Sakmann and colleagues (24) recently reported that M cells isolated from the guinea pig left ventricle show a smaller density of late \( I_{\text{Na}} \) compared with Epi and Endo cells.

![Fig. 5. Frequency dependence of late \( I_{\text{Na}} \) is similar across the ventricular wall. TTX-sensitive current was normalized to late \( I_{\text{Na}} \) during pulse 1 and plotted as a function of pulse number. Voltage protocol is shown at the top. A: late \( I_{\text{Na}} \) was characterized as the average current 30–35 ms after the start of a pulse. B: late \( I_{\text{Na}} \) was characterized as the average current 395–400 ms after the start of a pulse. Inset: comparison of TTX-sensitive current during pulse 1 (larger inward current) and the pulse 30 recorded in an M cell.](http://ajpheart.physiology.org/)

_AJP-Heart Circ Physiol • VOL 281 • AUGUST 2001 • www.ajpheart.org_
It is noteworthy that even in large ventricles (i.e., the dog) it is difficult to isolate Epi and Endo fractions that are not contaminated with transitional and M cells (18). It is possible that the distinctions demonstrated in this study underestimate the true differences of late $I_{\text{Na}}$ among the three cell types.

A second essential result of our study is that neither the frequency dependence assessed during a train of voltage-clamp pulses nor reactivation of late $I_{\text{Na}}$ showed any differences across the ventricular wall. A hallmark of the M cell AP is a steep rate dependence of APD$_{90}$. Our results suggest that late $I_{\text{Na}}$ contributes to this phenomenon. Because recovery of late $I_{\text{Na}}$ is similar in the three cell types, the absolute density of late $I_{\text{Na}}$ increases more in M cells, while cycle length is prolonged. The large density of late $I_{\text{Na}}$ in M cells also explains the greater abbreviation of the M cell AP in response to TTX (Fig. 1) and the greater prolongation in response to sea anemone toxin (ATX II), an agent that augments late $I_{\text{Na}}$. This greater late $I_{\text{Na}}$ reduces the net outward current present during the plateau of the M cell AP and contributes to the preferential prolongation of the M cell AP in response to potassium channel blockers (27, 28, 31, 32). The effectiveness of sodium channel blockers to reduce dispersion of repolarization further supports our results and the conclusion that transmural distribution of late $I_{\text{Na}}$ contributes to the rate dependence of ventricular APs (26, 29).

A TTX-sensitive calcium flux through sodium channels associated with activation of $\beta$-adrenergic receptors was found in rat ventricular myocytes (25). In the absence of external sodium, a calcium conductance inhibited by TTX has been found in both rat and guinea pig ventricular cells (1, 6, 13). TTX blocked this conductance over a limited range of voltages between $-65$ and $-30$ mV (1, 13). In our study, late $I_{\text{Na}}$ was measured in external solutions containing 2 mM CaCl$_2$ and 140 mM sodium methane sulfonate without activation of $\beta$-adrenergic receptors or protein kinase A. Substitution of monovalent cations on both sides of the membrane with the impermeant cation N-methyl-D-glucamine abolished all TTX-sensitive current, demonstrating the absence of a TTX-sensitive calcium conductance in the canine ventricle. This result is in agreement with those in which replacement of sodium with choline abolished all TTX-sensitive current in fetal or adult rat ventricular myocytes (7, 23).

Late $I_{\text{Na}}$ is caused by sustained sodium channel openings that continue well after the start of the depolarizing pulse. These late reopenings and bursting behavior occur with a low probability and have been associated with the slowly decaying whole cell $I_{\text{Na}}$ in ventricular myocytes from embryonic chicks, rabbits, guinea pigs, and rabbit cardiac Purkinje cells (12, 20, 22, 33). A larger late $I_{\text{Na}}$ associated with the LQT3 form of long Q-T syndrome in patients has been linked to a number of sodium channel mutations. Compared with wild-type sodium channels, these mutated channels have a greater percentage of sweeps exhibiting bursting behavior (8, 9). A majority of studies find that a single population of sodium channels underlies both the fast sodium conductance and late $I_{\text{Na}}$. Kiyosue and Arita (16) measured no appreciable differences between the conductance of channels active at the beginning of a depolarizing step and those exhibiting sustained reopenings. The transmural distribution of late $I_{\text{Na}}$ and fast sodium conductance as determined by measurements of $V_{\text{max}}$ is the same (3), consistent with a single population of channels of greater density in M cells. Patlak and Ortiz (22) concluded that a single population of sodium channels could function in different modes, each with a different inactivation rate. Similarly, Liu et al. (20) concluded that the same sodium channels that initiate the AP could also maintain the plateau. Saint et al. (23) measured rat whole cell currents and discovered that late $I_{\text{Na}}$ and fast $I_{\text{Na}}$
had a different voltage dependence and sensitivity to TTX, concluding that different channels were responsible for the two conductances. Although two populations of sodium channels may exist in the rat ventricle, a differential sensitivity to TTX can also arise from a state-dependent block of a single population of channels.

One source of potential error in our study is the transient overshoot of voltage that occurs at the beginning of each voltage step that triggers significant fast $I_{Na}$. We have shown that peak $I_{Na}$ remains very large in the presence of 10 μM TTX but that its decay is altered by TTX. If TTX also alters the decay of the voltage overshoot, then the time course of $I_{Ca}$ will be different before and after TTX application, and $I_{Ca}$ will be a component of the TTX-sensitive current. It is critical to understand that this contribution of $I_{Ca}$ to the TTX-sensitive current will be transient. To estimate the amplitude and duration of this contribution, we used the Luo and Rudy (21) model of cardiac ionic currents to establish the greatest possible contribution of $I_{Ca}$ to the TTX-sensitive current. We modeled the $I_{Ca}$ with a voltage step that included an overshoot that decayed with a time constant of 0.5 ms. The time course and amplitude of this overshoot was derived from measurements during a step to 0 mV in normal external sodium (35). To mimic TTX, we compared this to the $I_{Ca}$ one gets with a voltage step, which included an overshoot that decayed with a time constant of 0.25 ms. $I_{Ca}$ was smaller when the overshoot decayed more quickly, and this smaller $I_{Ca}$ caused an inward difference current during the test step. However, this difference current was always <10% of the total TTX-sensitive current measured at 30 ms in the present study, and this contribution decayed to zero 150 ms after the start of a step to 0 mV. The results of this modeling suggest that we have overestimated the density of the late $I_{Na}$ recorded 30 ms after the start of the test pulse but that measurements made after 295 ms accurately reflect late $I_{Na}$ density. Modeling also suggests that any heterogeneity in the density of calcium channels may contribute to the transmural distribution of TTX-sensitive current, but this contribution must be quite small, because TTX-sensitive current in M cells was 45% greater than Endo or Epi cells. $I_{Ca}$ in the Luo and Rudy model exhibits slower kinetics and a 10-fold greater density than our measurements of $I_{Ca}$ in the canine ventricle. Both of these factors should favor a larger contribution of $I_{Ca}$ to the TTX-sensitive current.

In an effort to mimic TTX-induced alteration of the voltage overshoot, we also investigated prepulse-induced modification of currents during the test pulse. The currents at the beginning of the test pulse were affected by the prepulse, and these changes outlasted the presumed alteration of the voltage profile. However, dramatic changes in this voltage profile during the beginning of the test step did not affect currents 30 or 295 ms later. Because this alteration of the voltage profile is not equivalent to the effects of 10 μM TTX, we fall back on the model of Luo and Rudy, which estimates that after 30 ms the upper limit of $I_{Ca}$ contribution to the TTX-sensitive difference current is 10%, and that this contribution is eliminated after 150 ms.

In summary, transmural voltage gradients are generated by differences in the time course of repolarization of the three cell types. In the midmyocardium, a higher density of sodium channels, a larger single-channel conductance, and/or a greater probability of reopening result in a larger late $I_{Na}$, which in M cells constitutes a greater fraction of total inward current at plateau voltages. Differences in $I_{Ks}$, $I_{Ca}$, and NaCa exchange also contribute to electrical heterogeneity within the canine ventricle, and these ionic differences can serve as a substrate for some arrhythmias. Studies using the canine arterially perfused wedge preparation demonstrate that these manifold ionic differences increase transmural dispersion of repolarization in response to agents that augment late $I_{Na}$ or inhibit the delayed rectifier current ($I_{Kr}$). Both of these perturbations serve to unmask inherent transmural differences and can lead to the development of the long Q-T syndrome. The $I_{Kr}$ blocker d-sotalol or agents that slow inactivation of $I_{Kr}$ like ATX II or anthopleurin A prolong the Q-T interval and induce extrasystoles capable of precipitating torsades de pointe. These agents preferentially prolong the duration of M cells and induce early afterdepolarizations in the midmyocardial region. Similar amplification of transmural dispersion of repolarization occurs when a $I_{Ks}$ blocker is combined with β-adrenergic activation to produce arrhythmias in the canine ventricular wedge (25, 26, 30). Taken together, these studies indicate the value of integrating voltage-clamp results in ventricular myocytes and electrophysiological studies in the arterially perfused wedge preparation and reveal the critical relation between electrical heterogeneity and generation of arrhythmias.

We thank Dr. Arthur Iodice for providing the dissociated myocytes. We also appreciate the expert technical assistance of Judy Hefferton, Robert Goodrow, and Di Hou.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-47678 (to C. Antzelevitch) and by the Masons of New York State and Florida.

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