Ranolazine Decreases Mechanosensitivity of the Voltage-Gated Sodium Ion Channel \textit{Na}_V1.5: A Novel Mechanism of Drug Action

Running title: \textit{Beyder et al.; Ranolazine decreases Na}_V1.5 stretch sensitivity

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Abstract:

**Background** - $\mathrm{Na}_V1.5$ is a mechanosensitive voltage gated sodium-selective ion channel responsible for the depolarizing current and maintenance of the action potential plateau in the heart. Ranolazine is a $\mathrm{Na}_V1.5$ antagonist with anti-anginal and anti-arrhythmia properties.

**Methods and Results** - Mechanosensitivity of $\mathrm{Na}_V1.5$ was tested in voltage-clamped whole cells and cell-attached patches by bath flow and patch pressure, respectively. In whole cells, bath flow increased peak inward current in both murine ventricular cardiac myocytes ($24\pm8\%$) and HEK cells heterologously expressing $\mathrm{Na}_V1.5$ ($18\pm3\%$). The flow-induced increases in peak current were blocked by ranolazine. In cell-attached patches from cardiac myocytes and $\mathrm{Na}_V1.5$-expressing HEK cells, negative pressure increased $\mathrm{Na}_V$ peak currents by $27\pm18\%$ and $18\pm4\%$ and hyperpolarized voltage dependence of activation by $-11\,\text{mV}$ and $-10\,\text{mV}$, respectively. In HEK cells, negative pressure also increased the window current ($250\%$) and increased late open channel events ($250\%$). Ranolazine decreased pressure-induced shift in the voltage-dependence ($\text{IC}_{50} 54\,\text{µM}$) and eliminated the pressure-induced increases in window current and late current event numbers. Block of $\mathrm{Na}_V1.5$ mechanosensitivity by ranolazine was not due to the known binding site on DIVS6 (F1760). The effect of ranolazine on mechanosensitivity of $\mathrm{Na}_V1.5$ was approximated by lidocaine. However, ionized ranolazine and charged lidocaine analog (QX-314) failed to block mechanosensitivity.

**Conclusions** - Ranolazine effectively inhibits mechanosensitivity of $\mathrm{Na}_V1.5$. The block of $\mathrm{Na}_V1.5$ mechanosensitivity by ranolazine does not utilize the established binding site, and may require bilayer partitioning. Ranolazine block of $\mathrm{Na}_V1.5$ mechanosensitivity may be relevant in disorders of mechano-electric dysfunction.

**Key words:** drugs; electrophysiology; ion channels; mechanics; myocytes
Introduction

Electro-mechanical coupling in the cardiac myocyte is vital for the generation of contraction. In turn, mechano-electric feedback (MEF) has an important regulatory role. Physiologic roles of MEF include stretch-related changes in sinoatrial node pacing frequency\(^1\), ventricular and atrial excitability\(^2\) and conduction velocity\(^3\). Acute mechano-electric dysfunction is a known pathophysiologic mechanism\(^4\), yet commonly, MEF pathologies are caused by chronic abnormalities of preload and afterload leading to stretch-related electrical disturbances\(^5\).

Redundancy in biological tissues exists to regulate MEF at cellular and molecular levels. Membrane tension opens stretch-activated channels (SACs), thus modulating electrical excitability\(^6\). However, molecular identities of mammalian non-selective cation stretch-activated channels remain elusive. Voltage-gated ion channels are also mechanically sensitive\(^7\). They are attractive MEF targets due to high density of expression and direct involvement in the coordination of electrical activity. \(\text{Na}_V\)1.5 is the principal voltage-gated sodium channel in cardiac myocytes\(^8\). \(\text{Na}_V\)1.5 is mechanosensitive and classic SACs blockers are known to inhibit this channel\(^9,10\). Acute stretch shifts \(\text{Na}_V\)1.5 voltage dependence of activation and inactivation\(^11\), resulting in accelerated kinetics\(^11,12\), increased peak currents\(^13\) and stabilization of inactivation\(^11\).

The piperazine-derivative ranolazine is a \(\text{Na}_V\)1.5 antagonist and an anti-anginal with anti-arrhythmic properties\(^14\) that shows promise for management of heart failure\(^15\). The molecular mechanism of ranolazine for ischemia could be through block of persistent (i.e., late) \(\text{Na}_V\)1.5 current\(^16\). Effects of ranolazine may also result from its hyperpolarizing shift in voltage-dependence of steady-state inactivation similar to the inactivated-state blocker lidocaine\(^14\). The function of lipid soluble drugs such as lidocaine\(^17\) and ranolazine may rely on partitioning into the lipid bilayer\(^18\). \(\text{Na}_V\) channels are modulated by the cytoskeleton\(^19\) & amphipaths\(^20\), and the
biochemical and mechanical states of the lipid bilayer\textsuperscript{21} impact mechanosensitivity of voltage-gated ion channels.

We hypothesized that ranolazine may modulate the mechanical behavior of Na\textsubscript{V}1.5. Our results show that the Na\textsubscript{V} channels native to cardiac myocytes or Na\textsubscript{V}1.5 transfected in HEK are mechanically sensitive. Ranolazine effectively blocks Na\textsubscript{V} and Na\textsubscript{V}1.5 mechanosensitivity, possibly via bilayer partitioning. Its mechanism is not due to binding to its established binding site.

**Methods**

**Adult Murine Cardiac Myocyte Dissociation**

Nine to eleven week old BALB/c mice (Harlan Sprague-Dawley, Indianapolis, IN) were used for the isolation of the ventricular cardiac myocytes as previously described\textsuperscript{22} and detailed in Supplementary Methods. The mice were maintained and the experiments were performed with approval from the Institutional Animal Care and Use Committee of the Mayo Clinic.

*Cell culture.* Human embryonic kidney 293 (HEK) cells were cultured and transfected as previously described\textsuperscript{11} (*Supplemental Methods*).

**Recording solutions & Pharmacology**

Recording solutions are detailed in Supplementary Methods. Ranolazine and lidocaine were diluted in bath or patch solution from 10 mM ethanol stocks. QX-314 was prepared daily from powder at 500 µM working concentration in pipette solution.

**Electrophysiology**

Electrodes were pulled using Sutter Instruments P-97 puller and coated with heat cured Dow Corning R6101 compound from Kimble KG-12 and Garner 8250 glass for whole-cell and patch
experiments, respectively. Axon 200A amplifier, Digidata 1322A, and Clampex 9 software were used for voltage-clamp and data acquisition.

**Whole cell electrophysiology**

A single 18 sweep, 90 sec pulse protocol was designed to measure peak current, voltage dependence, and kinetics of activation and inactivation. Cells were held at -120 mV, stepped to test pulses from -130 mV to 30 mV at 5 or 10 mV intervals for 3 sec, then to -120 mV for 0.1 msec, then to a second test pulse at -30 mV for 100 msec. The sampling rate was 20 kHz and intersweep interval was 5 sec.

**Cell-attached patches**

Electrodes were fire polished specifically for patch mechano-activation as previously published\(^1\). Seals were observed for 5 minutes for stabilization. For voltage-ladder protocols, a 1 Hz stimulation frequency was used with a P/4 protocol and no interpulse delay. Cells were held at -100 mV, and at each step a 4 msec pre-pulse to -204 mV accelerated recovery from inactivation before stepping in 10 mV increments from -140 mV to 10 mV for 80 msec per step to test activation and finally a step to 0 mV for 10 msec to test availability. A 10x average was obtained for each voltage step.

For single channel experiments, currents were digitized at 10 kHz and filtered at 2 kHz using a low-pass Bessel filter. Window protocol was a sequence of 300 msec long pulses at a specific HP, determined to be at the foot of the IV curve. Pressure was applied for 100 msec in the middle of each pulse. Late current protocol was a train of depolarizations from HP -100 mV to 0 mV for 200 msec with a 1.1 sec interpulse interval.

**Mechanical stimulation**

Mechanical stimuli consisted of flow and patch pressure for whole cell and cell-attached patches,
respectively. Flow of solution through a 0.7 mL elliptical bath chamber was calibrated at 10 mL/min. Specialized rapid pressure clamp was used to apply pressure (courtesy of Dr. Fred Sachs’ lab). Negative pressure produced patch stretch by displacement (Supplemental Movie 1) and was closely monitored during patch formation and pressure delivery for each of the protocols. Length of mechanical stimuli was <100 msec to minimize structural remodeling (Supplemental Methods).

Data Analysis

Whole cell and macroscopic patch data were analyzed in Pclamp 9 while single channel data were analyzed in QUB (www.qub.buffalo.edu). Voltage dependence and dose-response curves were fit in pClamp9 and Origin 8.61 (Supplemental Methods). For whole cell experiments significance was assigned when P<0.05 by a two-way repeated measures ANOVA with Bonferroni multiple comparisons posttest in GraphPad Prism 5 (Figure 1, Figure 6). For experiments on patches significance was P<0.05 by Student’s t-tests as specified in the text, and specifically two-tailed paired t-tests (Figure 4, Figure 5) and two-tailed two-sample equal variance t-tests (Figure 7, Figure 8) in Origin 8.61.

Results

Ranolazine inhibits the flow-induced increase in peak current in murine cardiomyocytes.

We used a 10 mL/min bath flow to simulate shear stress of voltage clamped murine cardiac ventricular myocytes in a control solution and then in the presence of 50 μM ranolazine. Flow of the control solution increased peak Na⁺ current from -28.0±4.7 pA/pF to -35.9±8.2 pA/pF, or by 24±8% (n=5, P<0.05, Figure 1A). In the same cells, 50 μM ranolazine decreased peak Na⁺ current from -28.0±4.7 pA/pF to -9.3±1.7 pA/pF, or by 68±3% (n=5, P<0.05). Ranolazine also
blocked the flow-induced increase of peak Na\(^+\) current from -9.3±1.7 pA/pF with ranolazine to -10.2±2.4 pA with ranolazine and bath flow, (6±7%, n=5, \(P>0.05\), **Figure 1A**). These data show that in cardiac myocytes ranolazine not only blocks peak Na\(_V\) current but also inhibits the response of Na\(_V\) channels to mechanical stimulation by bath flow.

**Ranolazine decreases Na\(_V\)1.5 peak current at rest and abolishes increase in current in response to bath flow in HEK cells**

Na\(_V\)1.5 is the predominant voltage-gated sodium channel in the adult murine\(^{25}\) and human\(^{8}\) cardiac myocytes. Next, we examined HEK cells heterologously expressing Na\(_V\)1.5 \(\alpha\)-subunit. Mechanical stimulation by flow increased Na\(_V\)1.5 maximum peak Na\(^+\) current from -137±16 pA/pF for control to -161±19 pA/pF for flow, or by 18±3% (n=9, \(P<0.05\), **Figure 1B**). Bath flow also accelerated the time constants of activation from 0.67±0.06 msec to 0.49±0.05 msec and inactivation from 0.87±0.08 msec to 0.77±0.07 msec (n=8, \(P<0.05\), **Supplementary Figure 1A**), consistent with published data\(^{26}\). Subsequent 10 min exposure to 50 \(\mu\)M ranolazine reduced maximum peak Na\(^+\) current from -137±16 pA/pF to -90±13 pA/pF, or by 37±5% (n=9, \(P<0.05\)) and inhibited the response to flow from -90±13 pA/pF with ranolazine to -100±14 pA/pF with ranolazine and bath flow, (10±3%, n=9, \(P>0.05\)). Block by ranolazine significantly interacted with mechanical activation of Na\(_V\)1.5, whereas the measurement of all other parameters such as voltage dependence and kinetics revealed no effect (\(P<0.05\) interaction for peak current, **Figure 1B**); and \(P>0.05\) for voltage dependence or kinetics, **Supplemental Figure 1A**). These data suggest that ranolazine can block current at rest and inhibit the mechanical activation of Na\(_V\)1.5 expressed in HEK cells similar to Na\(_V\) channels in cardiac myocytes.

**Ranolazine reduces pressure-induced hyperpolarizing shift in the voltage sensitivity of Na\(_V\)1.5**
To address the possibility that the effect of ranolazine could be dependent on the stimulus, we also tested the effect of ranolazine on Na\textsubscript{v}1.5 mechanosensitivity by pressure clamp on cell patches. Since ranolazine partitions readily into cell membranes (partition coefficient 1.53\textsuperscript{18}) we were able to add the drug to the bath solution with rapid effect (time constant \(\tau=19.4\pm1.6\) sec, \textbf{Supplemental Figure 2}). In Na\textsubscript{v}1.5 expressing HEK cells, addition of 50 \(\mu\text{M}\) ranolazine decreased peak currents by -32\pm8\% (\(n=4, P<0.05\), paired t-test), similar to the HEK whole-cell results above.

Before the application of ranolazine, the average peak patch current in the transfected cells was -78.6\pm69.2 pA (\(n=7\)). A -30 mmHg 100 msec long pressure pulse was applied at each voltage step during the subsequent ladder protocol of 17 steps from -140 to 30 mV by 10 mV increments. Patch pressure produced an increase in current amplitude of +18\pm4\% (\(n=7, P<0.05\), paired t-test) and accelerated kinetics at all steps as shown for -100 mV (dash dot), -50 mV (dot) and -30 mV (solid) (\textbf{Figure 2A}). Since pressure accelerated the kinetics of both activation and inactivation and increased peak currents, there was a significant difference current (\(I_{30}-I_0\)) (\textbf{Figure 2B}). For depolarization at the foot of the activation curve, pressure produced a significant increase in inward current, and a large inward difference current (dotted trace). For the large depolarization that is fully activating, the difference current was biphasic, with large early inward difference current followed by significantly decreased inward flux later in the pulse (solid trace).

In \textbf{Figure 2C} peak currents from step 1 and step 2 from a typical patch are plotted against voltage, showing voltage dependence of activation (squares) and availability (circles) at rest (0 mmHg) (black) and with -30 mmHg pressure (grey). Solid lines are Boltzmann fits of the peaks at the indicated voltages (solid lines). On average, for a -30 mmHg stimulus, shift in the voltage
dependence of activation ($\Delta V_{1/2a}$) was -10.1±1.5 mV (n=7, $P<0.05$, paired t-test) and shift in the voltage dependence of inactivation ($\Delta V_{1/2i}$) was -12.1±1.7 mV (n=6, $P<0.05$, paired t-test), peak Na$^+$ current change ($\Delta I_{peak}$) was 18±4% (n=7, $P<0.05$, paired t-test). We found no significant change in slopes ($\Delta dV_a=0.1±0.3$ mV, $\Delta dV_f=2.9±3.2$ mV, $P>0.05$ for both). These data demonstrate an increase in peak current, a shift in the voltage dependency of NaV1.5 current with pressure, suggesting that the channels will activate at more hyperpolarized voltages, which would make a cardiac myocyte more excitable.

After the addition of 50 μM ranolazine a -30 mmHg pressure failed to increase peak current and accelerate kinetics as for the control above, as exemplified in the raw traces (Figure 2D) and lack of difference current (Figure 2E). In the presence of 50 μM ranolazine, the half-point of the voltage dependence of activation ($V_{1/2a}$) did not shift with pressure (black is 0 mmHg, grey is -30 mmHg). Pressure resulted in an average $\Delta V_{1/2a}$ of -6.4±0.7 mV, which is smaller than with 0 μM ranolazine (n=5, $P<0.05$, two sample t-test) (Figure 2F). The peak current change ($\Delta I_{peak} = 14±4\%$) and shift in the voltage dependence of steady state inactivation ($\Delta V_{1/2i} = -8.7±0.5$ mV) with pressure were not statistically different from 0 μM ranolazine, (n=5, $P>0.05$ compared with 0 μM ranolazine, two-sample t-test). Also, no significant change was found for slope change ($\Delta dV_a=0.11±0.29$ mV, $\Delta dV_f=2.93±3.23$ mV, $P>0.05$, two-sample t-test).

We determined the potency of ranolazine inhibition of NaV1.5 mechanosensitivity. The voltage-dependence of activation ($V_{1/2a}$) and steady-state inactivation ($V_{1/2i}$) half-points from Boltzmann fits of IV curves for patches at rest (0 mmHg) and pressure (-30 mmHg) were obtained in the absence and presence of ranolazine at concentrations of 10, 50, 100, 300 μM (n≥3 per concentration). Pressure-dependence of the shift in the voltage-dependence of activation ($\Delta V_{1/2a}$) was fit to a dose-response curve with an IC$_{50}$ of 53.6 μM and hill slope $h=-0.01$ (Figure 3). Ranolazine also affected the mechanosensitivity of the voltage dependence of inactivation ($\Delta V_{1/2i}$) and peak current ($I_{30}/I_0$), but these effects were more difficult to quantify.
since ranolazine is known to affect both of these parameters at rest\textsuperscript{14} (Supplemental Figure 4). Thus, we elected to use $\Delta V_{1/2a}$ as an assay for pressure-effects of ranolazine on Nav1.5.

We next examined Nav pressure sensitivity and the ranolazine effect in a native system, using cell-attached patches in murine myocytes. In cell-attached patches from adult murine ventricular cardiac myocytes voltage dependent inward Na$^+$ current peaked at $-184\pm223$ pA and increased to $-263\pm372$ pA in response to $-20$ mmHg patch pressure, or by $27\pm18\%$ (n=5, $P<0.05$, paired t-test). Patch pressure hyperpolarized the voltage-dependence of activation, shifting $V_{1/2a}$ by $-11.4\pm6.7$ mV (n=5, $P<0.05$, paired t-test). Addition of 50 μM ranolazine decreased peak currents to $-65\pm35$ pA, or by 65% percent compared to no ranolazine controls (n=6, $P<0.05$, two-sample t-test). In the presence of 50 μM ranolazine negative pressure (-20 mmHg) produced a 66% smaller hyperpolarizing shift in the voltage-dependence of activation $V_{1/2a}$ of $-4.0\pm3.1$ mV compared to no ranolazine controls (n=5, $P<0.05$, two-sample t-test). These data from native myocytes support pressure sensitivity of Nav1.5 and inhibition of mechanosensitivity by ranolazine.

Ranolazine reduces pressure-induced increase in the Nav1.5 window current

Steady-state window is the area underneath the overlapping feet of activation and inactivation voltage dependence curves, where an appreciable portion of Nav1.5 channels is active (Figure 2C, D). We assessed pressure-dependent changes in window current activity using single channel recording. Cell-attached patches were stepped through a voltage ladder to determine the foot of activation. For the patch in Figure 4A this was $-40$ mV and averaged $-45\pm2.2$ mV (n=5). For each patch, we set the holding potential (HP) to the foot of activation and recorded 300 pulses of 300 msec at the left edge of the window. We applied pressure for 100 msec (bracket) every 200 msec. Single channel openings were idealized over 50 msec intervals before (black
bar) and during the -30 mmHg pressure step (grey bar). On average, the number of single channel open events increased from 85±40 at rest to 137±45 with a -30 mmHg pressure, representing a pairwise fractional increase (n=n30/n0) with pressure of 240±64% (n=5, P<0.05) (Figure 4Ai). Open channel lifetime did not change from 0.50±0.11 msec (control) to 0.54±0.13 msec (pressure), or a pairwise change (τ=τ30/τ0) by 28±29% (n=5, P>0.05) (Figure 4Aii). Since the single channel conductance does not change with pressure11, the significant increase in the number of open channel events produced a steady-state Na⁺ charge Q30/Q0 ([n30*τ30]/[n0*τ0]) increase of 260±31% (n=5, P<0.01, paired t-test). This significant increase in window current with pressure would depolarize the cell and likely make it more excitable.

Ranolazine (50 μM) reduced the pressure-induced increase in window current activity (Figure 4B). At HP of 40±6 mV (n=3), pressure failed to increase the number of single channel open events from 134±29 at rest to 147±44 with -30 mmHg pressure, or pairwise change of 9±14% in the window open channel numbers (n=3, P>0.05) (Figure 4Bi). Open channel lifetimes also did not change from 0.66±0.10 msec at rest to 0.68±0.057 msec, a pairwise change of 4±6% (n=3, P>0.05) (Figure 4Bii). The result was no change in steady-state Na⁺ charge (14±20%, n=3, P>0.05). These results suggest that pressure-induced increase in window current at resting potential is abolished by ranolazine.

Ranolazine reduces pressure-induced increase in the Na\textsubscript{v}1.5 late current open channel events

Na⁺ late current is the small steady-state flux during the action potential plateau. Late current abnormalities have been implicated in cardiac pathologies such as LQT327, ischemia28 and heart failure15, and are thought to be the therapeutic target for ranolazine16. In a cell-attached configuration the late current is comprised of sporadic single channel events (Figure 5A). We
used a 200 msec step depolarization from -100 mV to 0 mV, and analyzed single channel events in the last 100 msec from individual patches at rest (0 mmHg, data not shown) and then using a -30 mmHg ramp pressure applied to the same patch (Figure 5A). Compared to the late single channel events at rest (19±5), -30 mmHg pressure produced an increase in event number (42±10), or a 235±44% pairwise increase (n=4, P<0.05) (Figure 5Ai). Single channel open lifetime trended to a decrease from control (0.61±0.11 msec) to pressure (0.37±0.07 msec), or a pairwise decrease by 71±18% (n=4, P>0.05) (Figure 5Aii). Assuming no change in single channel conductance11, total late steady-state Na⁺ charge did not change (19±70%, n=4, P>0.05). Ranolazine (50 μM) abolished the pressure-induced changes in late current (Figure 5B). The single channel event number did not change from rest (40±14) to negative pressure (28±5), or a pairwise change of 22±30% (n=5, P>0.05) (Figure 5Bi). Pressure also did not change open channel lifetime from 0.52±0.10 msec at 0 mmHg to 0.61±0.02 msec at -30 mmHg, or a pairwise change of 27±23% (n=5, P>0.05) (Figure 5Bii). Total late steady-state Na⁺ charge was not changed (34±79%, n=5, P>0.05). Thus, the pressure-induced increase in late single channel opening numbers is abolished with ranolazine.

**F1760A mutation decreases Naᵥ1.5 peak current block by ranolazine, but does not eliminate mechanoinhibition**

Ranolazine block of Naᵥ1.5 requires the putative local anesthetic binding site on DIVS6 F176029. Hence, we investigated whether inhibition of mechanosensitivity by ranolazine could be altered by site-directed mutagenesis of residue F1760. In whole-cell voltage clamped HEK, F1760A Naᵥ1.5 peak currents were insensitive to 50 μM ranolazine, as current densities changed only 2±6%, from -195±29 pA/pF in control to -201±32 pA/pF with drug (n=6, P>0.05) (Figure 6). Nevertheless, F1760A Naᵥ1.5 channels appeared to retain mechanosensitivity like wild-type
Nav1.5, as F1760A currents increased 18±5% from -195±29 pA/pF to -228±32 pA/pF in response to flow of drug-free solution (n=6, P<0.05). Moreover, inhibition of flow response was also retained in F1760A channels like wild-type; F1760A peak Na⁺ currents were not inducible by flow of ranolazine solution, changing only 8±4% from -201±32 pA/pF with flow off to -213±30 pA/pF with flow on (n=6, P>0.05).

We confirmed these findings in cell-attached patches. At rest (0 mmHg), while the application of ranolazine decreased wild-type Nav1.5 current by 32±8% (n=4), the peak currents of F1760A Nav1.5 channels in presence of 50 μM ranolazine were decreased by only 11±9% (n=4, P<0.05 compared to wild type current) (Figure 7A). The F1760A Nav1.5 channels were mechanosensitive to a similar extent as the wild-type channels, with a -30 mmHg pulse producing ΔV1/2a of -10.8±2.5 mV (n=4), which was significantly reduced by 50 μM ranolazine to ΔV1/2a -4.7±1.8 mV (n=7) (P<0.05) (Figure 7B).

The above whole cell and cell-attached patch data confirm that F1760 is required for significant ranolazine block of NaV1.5 current at rest29. Yet, F1760A Nav1.5 retains mechanosensitivity, and ranolazine continues to inhibit F1760A NaV1.5 mechanosensitivity to a similar extent as in the wild-type NaV1.5. These results suggest that the ranolazine inhibition of NaV1.5 mechanosensitivity is not due to binding to F1760.

Ranolazine inhibition of mechanosensitivity of NaV1.5 may require drug partitioning into the lipid bilayer

Ranolazine has a pKa of 7.17, so at pH 5 the positively charged form of ranolazine is roughly 100-fold more concentrated than the neutral form. We used pH 5 and 50 μM ranolazine in the pipette only for these experiments as bath acidity is known to affect multiple cellular processes. For a -30 mmHg pressure, wild-type NaV1.5 channels shifted the half-point of voltage sensitivity...
of activation $\Delta V_{1/2a}$ by $-7.4\pm1.0$ mV (n=7) at pH 5 and $-10.1\pm1.5$ mV (n=5) at pH 7 ($P>0.05$).

With the pipette containing 50 $\mu$M ranolazine and pH 5 solution, pressure produced a shift of $\Delta V_{1/2a}$ of $-5.2\pm1.7$ mV (n=7), compared to $-7.4\pm1.0$ mV (n=7) without ranolazine ($P>0.05$) (Figure 8A). Thus, it appears that ionized ranolazine does not inhibit the shift in $\Delta V_{1/2a}$ at pH 5.

Since the ionized form of ranolazine does not significantly partition into the hydrophobic core of the bilayer, these data suggest that ranolazine inhibition of Na\textsubscript{v}1.5 mechanosensitivity may require bilayer partitioning.

The similarities of ranolazine and local anesthetics with respect to the overlapping binding sites and chemical properties (structure, logP, pKa) suggested that the mechanisms of action may also overlap. It is known that local anesthetics that exist in neutral forms, such as lidocaine, partition significantly into the hydrophobic core of the bilayer. Cell-attached patches were exposed to 50 $\mu$M lidocaine by addition to the bath as before. Lidocaine (50 $\mu$M) also blocked mechanosensitivity of Na\textsubscript{v}1.5, with pressure-induced $\Delta V_{1/2a}$ -5.6\pm1.6 (n=5) (Figure 8B). This $\Delta V_{1/2a}$ is smaller than $-10.1\pm1.5$ mV shift for the controls without lidocaine (Figure 8A).

Thus, it appears that Na\textsubscript{v}1.5 mechanosensitivity block is not specific for ranolazine. We next used the permanently charged lidocaine homolog QX-314 to determine the need for bilayer partitioning for mechanosensitivity block. QX-314 is membrane impermeable therefore we added 500 $\mu$M QX-314 to the pipette solution. In the presence of QX-314, Na\textsubscript{v}1.5 mechanosensitivity persisted with a $\Delta V_{1/2a}$ $-12.0\pm2.9$ mV (n=3, $P>0.05$ compared to lidocaine) (Figure 8B).

**Discussion**
In this study we demonstrate Na\textsubscript{V} mechanosensitivity in adult murine ventricular cardiac myocytes and in HEK cells transfected with Na\textsubscript{V}1.5. We describe multiple effects of mechanical stimulation on Na\textsubscript{V}1.5 function and demonstrate effective inhibition of Na\textsubscript{V}1.5 mechanosensitivity by ranolazine and lidocaine. Furthermore, we establish that the inhibition of Na\textsubscript{V}1.5 mechanosensitivity by ranolazine does not require the established binding site, but appears to require bilayer partitioning.

Mechanical stimulation in cardiac myocytes and Na\textsubscript{V}1.5-transfected HEK cells increased peak Na\textsubscript{V} current, accelerated kinetics of activation and inactivation and in patches hyperpolarized the half-points of voltage-dependence of activation (V\textsubscript{1/2a}) and inactivation (V\textsubscript{1/2i}), thereby left-shifting the window current (Figure 1, Figure 2A-C). At the single channel level mechanical stimulation of Na\textsubscript{V}1.5 increased total window current at hyperpolarized resting potentials (Figure 4A) but not late current during depolarization to 0 mV (Figure 5A). These results suggest that Na\textsubscript{V}1.5 may contribute to MEF. In a cardiac myocyte membrane depolarization is the most consistent effect of stretch\textsuperscript{30}. We showed two mechano-induced effects that would contribute to membrane depolarization: hyperpolarization of the Na\textsubscript{V}1.5 window current and a large increase in total Na\textsuperscript{+} charge density for small depolarizations (Figure 2B, C). On the other hand, for large depolarizations mechano-induced acceleration in inactivation led to a relative decrease in Na\textsuperscript{+} influx following the upstroke (Figure 2B). This may contribute to the early shortening of the action potential duration (APD) by stretch as previously shown\textsuperscript{31}. However, stretch prolongs latter portions of APD\textsuperscript{30}, which may predispose to development of secondary depolarization\textsuperscript{5}. Stretch accelerates Na\textsubscript{V}1.5 inactivation and does not alter late current, so it is unlikely that Na\textsubscript{V}1.5 is involved in stretch-dependent ADP prolongation. Potential mechanisms include inhibition of K\textsuperscript{+} selective\textsuperscript{32} or non-selective SACs\textsuperscript{33}. In contrast, mechano-
induced acceleration of \( \text{Na}_V \text{1.5} \) inactivation kinetics may protect from excessive stretch-dependent action potential prolongation. A recent study of the classic LQT3 \( \text{Na}_V \text{1.5} \) mutations that have defective inactivation showed that pressure failed to accelerate inactivation in proportion to activation\(^{34} \), suggesting that pressure-induced acceleration of \( \text{Na}_V \text{1.5} \) inactivation may be a protective MEF response.

Ranolazine inhibited \( \text{Na}_V \text{1.5} \) mechanosensitivity in both cardiac myocytes and HEK cells. The drug diminished the mechano-induced increase in peak current, shift in voltage sensitivity (Figure 1, Figure 2D-F), increases in the window current (Figure 4B) and late current open channel event numbers (Figure 5B). Ranolazine inhibition of \( \text{Na}_V \text{1.5} \) mechanosensitivity would indicate multiple effects on stretch-dependence in a cardiac myocyte. Most obvious is that loss of the hyperpolarizing shift in voltage-dependence of activation and window current would decrease the excitability with stretch. Block of stretch-dependence of \( \text{Na}_V \text{1.5} \) inactivation would be more complex. We confirm that ranolazine left-shifts \( V_{1/2i} \) at rest\(^{35} \) (Supplemental Figure 3) but we also show that it inhibits the mechano-induced leftward \( V_{1/2i} \) shift (Supplemental Figure 4A). Since \( V_{1/2i} \) shift is dose-dependent with respect to stretch\(^{11} \), the final result of \( V_{1/2i} \) position depends on doses of stretch and drug and would be difficult to predict.

The IC\(_{50} \) of ranolazine block of \( \Delta V_{1/2a} \), the most sensitive pressure-sensitive parameter, was 54 \( \mu \text{M} \) (Figure 3). This value is about five-fold higher than the accepted plasma concentration\(^{36} \).

However, there are at least two reasons not to dismiss the inhibitory effect of ranolazine on mechanosensitivity of \( \text{Na}_V \text{1.5} \). First, we do not know the true mechanical environment of the channels in situ. Previous data suggest that \( \text{Na}_V \text{1.5} \) mechanosensitivity is likely mediated by a combination of the lipid bilayer\(^{20} \) and the cytoskeleton\(^{26} \). In the native setting, \( \text{Na}_V \text{1.5} \) resides
within and associates with other mechano-relevant elements. \(\text{NaV}_1.5\) channels are localized within T-tubules\(^{37}\) and caveolin-3 rich rafts\(^{38}\), which are membrane structures with high intrinsic curvature, and tend to be areas of mechanosensitivity\(^{39}\). The \(\text{NaV}_1.5\) channels also make extensive intracellular connections\(^{40}\). Some of the \(\text{NaV}_1.5\) associating proteins, such as ankyrin\(^{41}\), syntrophin\(^{42,43}\) and telethonin\(^{44}\), are known to be proteins involved in cellular mechanosensitivity. Dysfunctional interaction of \(\text{NaV}_1.5\) with associating proteins is known to contribute to pathology\(^{43,45}\), so these connections may serve to further focus mechanical force at the channel\(^{45}\). While we used a -30 mmHg stimulus, which is at the lower end of the typical pressures used to study SACs\(^6\), the degree of mechanical stimulation in situ remains to be determined. Second, the membrane concentration of the drug likely differs from the plasma concentration. The neutral form of ranolazine is the effective inhibitor of \(\text{NaV}_1.5\) mechanosensitivity and it partitions substantially into the bilayer hydrophobic core. Membrane diffusion\(^{46}\) and partition coefficients\(^{47}\) increase with the extra energy, whether in the form of stretch or temperature. As our experiments were carried out at room temperature we expect that effective membrane concentration will further increase at physiologic temperature.

Our results shed some light on the mechanism of \(\text{NaV}_1.5\) mechanosensitivity. Inhibition by ranolazine does not require the putative binding site on DIVS6 (Figure 7) and neutral lidocaine and ranolazine inhibit, but charged ranolazine and permanently charged lidocaine analog QX-314 do not impact mechanosensitivity (Figure 8). This suggests the importance of drug partitioning into the lipid bilayer, but does not rule out other protein binding sites or cytoskeletal involvement. Previous studies support the importance of the lipid-protein interface in \(\text{NaV}\) function\(^{20}\) and voltage-gated channel mechanosensitivity\(^{21}\). Many outstanding questions
remain regarding the mechanism of NaV1.5 mechanosensitivity, but it very likely requires a combination of the associating proteins, membrane domains and protein-lipid interface.

This study demonstrates the mechanosensitivity of NaV1.5 in both native cardiac myocytes and a heterologous system. We establish drugs such as ranolazine, lidocaine and their analogs as pharmacologic tools for inhibition of NaV1.5 mechanosensitivity and suggest potential mechanisms. Further exploration of these compounds in disorders associated with MEF may be warranted. Examples include novel heart failure therapy and atrial fibrillation related to stretch of pulmonary veins, recently shown to be suppressed by ranolazine.

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Conflict of Interest Disclosures: None.

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Figure Legends:

**Figure 1.** Ranolazine blocks mechanosensitive response and peak currents of Na⁺ channels in murine cardiac myocytes and HEK cells transfected with Naᵥ1.5. **Left, A,** Representative Na⁺ currents recorded by whole cell voltage-clamp from murine cardiac myocytes and **B,** HEK cells transfected with Naᵥ1.5, elicited by stepping to -30 mV from -120 mV before *(black traces, Flow OFF)* or during *(grey traces, Flow ON)* bath flow, produced by rinsing solution through the recording chamber at 10 mL/min in the absence *(Control, 0 µM)* or presence *(Ranolazine, 50 µM)* of drug. **Right,** Average peak current densities in response to flow of solution without
(filled symbols) or with (empty symbols) ranolazine (n=5; *P<0.05 compared to Flow OFF, †P<0.05 compared to 0 μM ranolazine, and P<0.05 interaction between flow and ranolazine blockade by two-way repeated measures ANOVA with Bonferroni multiple comparisons posttest).

Figure 2. Ranolazine blocks pressure-induced increase in peak current and hyperpolarizing shift of the voltage-dependence of activation and inactivation. A, B, C are controls and D, E, F are ranolazine (50 μM). A & D, single patch average Na⁺ currents elicited by stepping to -100 mV (dash dot) -50 mV (dot), -30 mV (solid) from -140 mV at 0 mmHg (black) and at -30 mmHg pressure (grey). First peak is activation and second peak is inactivation (availability). B & E, same patch difference currents (I₃₀ – I₀) shown for -50 mV (dot black) and -30 mV (solid black). C & F, peak current-voltage (IV) for this patch at 0 mmHg (black) and -30 mmHg (grey), with activation (boxes) and inactivation (circles). Solid lines are Boltzmann fits of 0 mmHg (black) and -30 mmHg (grey).

Figure 3. Ranolazine blocks mechanosensitivity of Naᵥ1.5 in a concentration-dependent manner. Bar graph is shift in the voltage dependence of activation (ΔV₁₂a) for 0 μM ranolazine and scatter plot is ΔV₁₂a versus increasing ranolazine concentrations. Solid line is a dose-response fit with IC₅₀ of 53.6 μM.

Figure 4. Ranolazine inhibits pressure-induced increase in window current. A are controls and B are ranolazine (50 μM). A & B, single channel Na⁺ window current at HP -40 mV typical (black) and overlaid first fifty 300 msec traces (grey) at 0 mmHg and -30 mmHg (bracket).
Single channel activity idealized before (black bar) and during (grey bar) the pressure pulse. Bar graphs show for -30 mmHg compared to 0 mmHg percent change of open channel event number (#30/#0 x 100) for control (Ai) and 50 μM ranolazine (Bi), and open channel lifetime (τ30/τ0 x 100) for control (Aii) and 50 μM ranolazine (Bii) (n=5, *P<0.05; paired t-test for events between 0 mmHg and -30mmHg).

**Figure 5.** Ranolazine inhibits pressure-induced increase in late open channel event number. A are controls and B are ranolazine (50 μM). A & B, single channel Na⁺ late current typical (black) and overlaid first fifty (grey) 200 msec long depolarizing pulses to 0 mV from HP -100 mV for ramp to -30 mmHg (bottom). Single channel activity in the last 100 msec of late current activity analyzed (grey bars). Bar graphs show for -30 mmHg compared to 0 mmHg percent change in open channel event number (#30/#0 x 100) for control (Ai) and 50 μM ranolazine (Bi), and for open channel lifetime (τ30/τ0 x 100) for control (Aii) and 50 μM ranolazine (Bii) (n=4, *P<0.05; paired t-test for events between 0 mmHg and -30mmHg).

**Figure 6.** Ranolazine blocks a mechanosensitive response but does not reduce peak currents of Na\textsubscript{v}1.5 F1760A expressed in HEK cells. Left, Na⁺ currents elicited by stepping to -30 mV from -120 mV before (black, Flow OFF) or during (grey, Flow ON) bath flow, produced by rinsing solution through the recording chamber at 10 mL/min in the absence (Control, 0 μM) or presence (Ranolazine, 50 μM) of drug. Right, Average peak current densities of Na\textsubscript{v}1.5 F1760A in response to flow of solution without (filled symbols) or with (empty symbols) 50 μM ranolazine (n=6; *P<0.05 compared to Flow OFF, P>0.05 compared to 0 μM ranolazine, and
P>0.05 interaction between flow and ranolazine blockade by two-way repeated measures ANOVA with Bonferroni multiple comparisons posttest).

**Figure 7.** Ranolazine block of mechanosensitivity is independent of F1760. **A,** the fractional decrease in peak current \([I_{\text{ctr}}-I_{\text{ran}}]/I_{\text{ctr}}\) obtained from peak of IV curves in response to 50 μM ranolazine for wild-type NaV1.5 (left) and F1760A (right) (n=4; *P<0.05; two-sample t-test). **B,** mechanosensitivity of F1760A shown as a shift in half-point of voltage-dependence of activation (ΔV1/2a) without ranolazine and significantly reduced with 50 μM ranolazine (n=4-7, *P<0.05; two sample t-test).

**Figure 8.** Neutral ranolazine and lidocaine block mechanosensitivity of NaV1.5. **A,** In cell-attached patches, pressure-induced ΔV1/2a with wt NaV1.5 in Ringer solution at pH 7.4 was not statistically different from ΔV1/2a when the pipette solution contained Ringer solution at pH 5 (n=5-7, P>0.05; two-sample t-test). ΔV1/2a was also not statistically different for pH 5 solution and ranolazine (50 μM) in pH 5 solution (n=7, P>0.05; two-sample t-test). **B,** Shift in V1/2a with pressure with lidocaine (50 μM) is significantly less than with QX-314 (500 μM) (n=3-5, *P<0.05; two-sample t-test).
Ranolazine Decreases Mechanosensitivity of the Voltage-Gated Sodium Ion Channel Nav1.5: A Novel Mechanism of Drug Action

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Supplemental Material
Supplementary Methods:

**Adult Murine Cardiac Myocyte Dissociation:** Isolation of cardiac cardiomyocytes was as in with slight modifications\(^1\). Nine to 11 week-old male BALB/c mice were anesthetized with pentobarbital. After mechanical ventilation, the aorta was cannulated *in situ* and the heart rapidly excised and retrogradely perfused at 2-3 ml/min (~80-100 mmHg) for 5 min with Tyrode solution (137 mM NaCl, 5.4 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM HEPES, and 10 mM glucose), followed by 1 min with a 'low-calcium' medium (100 mM NaCl, 10 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 5 mM MgSO\(_4\), 20 mM glucose, 50 mM taurine, 10 mM HEPES) supplemented with 0.13 mM CaCl\(_2\) and 2.1 mM EGTA. Enzymatic digestion was started by 15 min perfusion with 'low-calcium' medium supplemented with 1% BSA, 0.2 mM CaCl\(_2\), collagenase (type IV, 22 U/ml; type II, 7U/ml; Worthington) and pronase (100 µg/ml; Serva). Perfusion solutions were oxygenated by bubbling with 95%/5% O\(_2\)/N\(_2\). The left ventricle was then removed, cut into pieces (~3x3 mm) and incubated at 37°C for 5 min in the enzyme solution with gentle stirring. Cardiac myocytes were finally mechanically dissociated by gentle pipetting tissue pieces. Cells were centrifuged at 500 r.p.m. for 1 min and the pellet was washed twice in 'low-calcium' medium supplemented with 0.2 mM CaCl\(_2\) ('wash'). Cell pellet was finally suspended in 'wash' and kept at room temperature until use. All solutions were kept at 37°C with pH 7.3.

**HEK Cell culture:** Cells were grown in T25 flasks in Minimum Essential Medium, 10% horse serum, non-essential amino acids, sodium pyruvate and penicillin-streptomycin (Invitrogen, Carlsbad, CA). Cells were grown to about 75% confluency. One day prior to the experiments, flasks were transfected with SCN5A encoding hH1 isoforms c1 or c3, and with green fluorescent
protein (GFP) pEGFP-C1 (Clontech, Palo Alto, CA), using Lipofectamine 2000 reagent and OPTI-MEM I reduced serum medium (Invitrogen, Carlsbad, CA). On the morning of experiments, cells were trypsinized and resuspended in Ringers solution and plated onto the recording chamber 15 minutes prior to the initiation of experiments.

**Recording Solutions:**

*Whole cell electrophysiology:* The intracellular solution contained (in mM): 125 CH₃SO₃⁻, 35 Cl⁻, 145 Cs⁺, 5 Na⁺, 5 Mg²⁺, 5 HEPES, 2 EGTA. pH was adjusted to 7.0 with CsOH. The extracellular solution contained (in mM): 159 Cl⁻, 139 Cs⁺, 15 Na⁺, 4.7 K⁺, 2.5 Ca²⁺, 10 HEPES, and 5.5 glucose. pH was adjusted to 7.4 with NaOH. The predicted whole cell liquid junction potential of 7.4 mV was subtracted during whole cell analysis.

*Cell-attached patches:* For patch experiments on HEK cells, bath solution was high-K for macroscopic recordings and high-Cs for single channel recordings. High-K solution contained (in mM): 149 K⁺, 2.5 Ca²⁺, 5 HEPES, 5.5 glucose. High-Cs solution contained (in mM): 139 Cs⁺, 15 Na⁺, 4.7 K⁺, 2.5 Ca²⁺, 10 HEPES, 5.5 glucose. The cardiac myocyte bath solution was the “low calcium” solution 100 mM NaCl, 10 mM KCl, 1.2 mM KH₂PO₄, 5 mM MgSO₄, 20 mM glucose, 50 mM taurine, 10 mM HEPES. Pipette solution was (in mM) 149 Na⁺, 4.7 K⁺, 2.5 Ca²⁺, 5 HEPES. GdCl₃ (10µM) was added to the pipette solution on the day of experiments, except when ranolazine, QX-314 and pH 5.0 were used in the pipette. This concentration of GdCl₃ was previously established to provide sufficient blockade of the endogenous stretch channels, without a significant effect on mechanosensitivity of Naᵥ1.5 channels². For pH 5.0 experiments adjustments were made with NaOH. Osmolality for all solutions was 290 mmol/kg.
Mechanical Activation of Voltage-clamped Currents:

Whole cell electrophysiology with bath flow: After a seal and whole cell access were obtained, control currents were recorded in ranolazine-free solution until a stable baseline was established. Next, an elliptical 0.7 mL bath was flushed with ranolazine-free solution at a rate of 10 mL/min for the duration of the 90 s recording to obtain flow data as a paired control. Then, extracellular solution plus ranolazine (premixed to 50 µM) was washed into the chamber at the conclusion of the flow record. Previous work has shown that mechanical activation of NaV1.5 is reversible after minutes. Therefore, ranolazine solution was allowed to incubate on cells for 10 min before the ranolazine data was recorded. Finally, the bath was flushed with ranolazine solution at 10 mL/min to determine the effect of ranolazine on mechanosensitivity of NaV1.5 by flow. In alternate experiments, ranolazine data were collected first before paired controls 10 min later.

Cell-attached patch with direct stretch by pipette pressure: Due to the mechanical remodeling of patches with prolonged stretch pulses we used a rapid pressure clamp (rise time to -30 mmHg in 5 msec) stretch steps were limited to 100 msec during activation/inactivation steps only. Seal history is important for such experiments, therefore we recorded the pressures required for patch formation. Patches were typically formed at <5 mmHg. Patches with seals that were formed at >10 mmHg were discarded. Pressure was stepped to the desired level for a few seconds prior to protocol execution, and then stepped back to 0 mmHg between successive datasets.

For experimental systems that aim to combine voltage- and mechano-clamp, application of pressure to a voltage-clamped membrane patch is the most reproducible stimulus available. Determination of the actual stimulus at the patch is complicated since the geometry is not
spherical, and the patch anatomy is inhomogeneous and changes in time\(^4\). Therefore, we focused on differential effects within the same patch\(^5\).

**Data Analysis:**

**Whole cell electrophysiology:** Peak currents during the first series of test pulses determined the voltage dependence of activation, whereas peak currents at test pulse 2 versus the voltages of test pulse 1 determined the voltage dependence of inactivation. Peak currents normalized to the equation \(I_{\text{norm}}=100(I_1)/I_{\text{peak}}\) were fit with a sigmoid 3-parameter curve: \(y=1/(1+\exp((x_0-x)/b))\), where \(x_0\) is \(V_{1/2}\), the voltage of half-activation or half-inactivation. Currents activated over the first 50 ms were fit with a 3-term weighted exponential equation: \(f(t) = K_0(\sum f_i \exp(-t/\tau_i))\), in which three time constants (\(\tau_i\)), represent one activation and two inactivation states of Na\(_V\)1.5. Peak currents are expressed as a fraction of cell capacitance (pA/pF).

**Cell-attached patches:** Voltage dependence of activation and steady-state inactivation were fit using a two state Boltzmann model (\(I=A/[1+\exp((V_{1/2}-V)/dV)]\)) where normalized peak currents (I) were plotted against applied voltage. Bath exchange time response was fit using an exponential decay function (\(I=I_o+A\exp(t/\tau)\)), where peak currents from successive steps were plotted against time (t). Two dose-response functions were used. A single dose-response function \(y=A_1+(A_2-A_1)/(1+10^{(\log(x_o)-x)*h})\), where \(x\) was ranolazine concentration, \(A\) were constants, \(x_o\) was IC\(_{50}\) and \(h\) was the hill slope. Double dose-response function \(y=A_1+(A_2-A_1)[p/(1+10^{(x_{0,1}-x)*h_1})+(1-p)/(1+10^{(x_{0,2}-x)*h_2})]\) was used to obtain two IC\(_{50}\) values (\(x_{0,1}\) and \(x_{0,2}\)) and two hill slopes (\(h_1\) and \(h_2\)).
**Single channels:** First, acquired traces were chopped into segments of desired length. Typical baseline and open channel amplitudes were input into a two-state kinetic model. Log-likelihood minimization was used to idealize the single channel events\(^6\).
Supplementary Video 1. Negative pressure stretches the patch. Transfected cell was patch-clamped and suction-induced changes were imaged with Olympus IX70 using DIC optics through 60x (NA 0.90) long-working distance objective and recorded as time-sequence images (10 fps) using a CCD camera (Hamamatsu Photonics, Japan). Suction was applied through the side-port of the patch electrode holder. Pressure was stepped in 100 msec intervals from 0 mmHg to -30 mmHg.
Supplementary Figure 1. Effects of flow of solution with or without ranolazine on the voltage dependence and kinetics of Na⁺ channels in HEK293 cells transfected with SCN5A F1760 (wild-type NaV1.5) or F1760A. A-B, Effects of bath flow at a rate of 0 (OFF) or 10 mL/min (ON) of solution containing 0 (filled circles) or 50 µM ranolazine (empty circles) on the voltage dependent and kinetic properties of Na⁺ channels in HEK293 cells transfected with NaV1.5 F1760 (A) or F1760A (B). Columns, from left to right, V₁/₂ of steady-state activation, V₁/₂ of steady-state inactivation, time constant (τ) of activation, fast time constant of inactivation (τ₁), and slow time constant of inactivation (τ₂). (n=6-9; *P<0.05 compared to 0 mL/min bath flow, †P<0.05 compared to 0 µM ranolazine, and P>0.05 interaction between bath flow and ranolazine blockade by two-way repeated measures ANOVA with Bonferroni multiple comparisons posttest).
Supplementary Figure 2. Ranolazine can be applied to cell-attached patches using bath exchange. Cell-attached patches were voltage-clamped and stepped at 1Hz to 0 mV from HP=-120 mV and using a -204 mV pre-pulse to recover from inactivation. Peak currents were monitored before and after application of Ranolazine to the bath. In this example, 300 μM Ranolazine was applied to the bath at 30 seconds and peak current reduced by ~7.5 pA within seconds. When fit to an exponential decay function (solid green line) (t from 0 to 200 sec), τ was 19.4±1.6 sec. Average time constant (τ) was 20.7±5.5 sec (n=5). Bath exchange with control solution did not produce such changes.
Supplementary Figure 3. A typical cell-attached patch with and without ranolazine (100 μM). Peak current versus voltage for activation (squares) and steady-state inactivation (circles) were obtained at rest (0 mmHg) without ranolazine (black traces) and with 100 μM ranolazine (red traces). A, peak current decreased from 228 pA for control to 59 pA for ranolazine. B, voltage dependence of activation did not change from $V_{1/2a}$ of -46 mV for control to -46 mV for ranolazine, while voltage dependence of steady-state inactivation $V_{1/2i}$ was hyperpolarized from -66 mV to -71 mV. Slopes of activation ($dV_a$) changed from 4.3 mV for control to 4.6 mV for ranolazine and inactivation ($dV_i$) from 8.0 mV for control to 10.0 mV for ranolazine.
Supplementary Figure 4. Scatter plots (n≥3 per data point) showing ranolazine concentration versus shift in the voltage-dependence of inactivation (ΔV_{1/2i}) (A), and fractional change in peak current (I_{30}/I_{0}) (B). Solid lines are data fits using single dose-response functions (B), and biphasic dose-response (A).

A) The shift of voltage-dependence of inactivation (ΔV_{1/2i}) in response to ranolazine was biphasic. At the smaller concentrations of ranolazine (10 – 100 µM), we observed a decrease in ΔV_{1/2i} of similar amplitude to ΔV_{1/2a}. However, at the highest concentration tested (300 µM), the shift in V_{1/2i} exceeded that for the controls. This behavior was fit using a biphasic dose-response function, with IC_{50}s of x_{0,1}=60.0 and x_{0,2}=120.0 µM and hill slopes of h_{1}=0.02 and h_{2}=-0.01. The two IC_{50} values match the mechanically sensitive changes of V_{1/2a} (x_{0,1}) above and previously published IC_{50}=135 µM for peak current inhibition (x_{0,2})^7. A possible explanation is that, the biphasic effect is due to the fact that ranolazine stabilizes inactivation independently of pressure, and at high concentrations these effects overcome those of mechano-inhibition (see Supplementary Figure 3 above).

B) Analysis of peak current decrease with pressure was complicated by the very small residual current at high ranolazine concentrations and the variability of response. Peak current concentration-response was fit to a dose-response function, yielding IC_{50} of 123.1 µM and a hill slope of h=-0.005.
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