Ranolazine Reduces Neuronal Excitability by Interacting with Inactivated States of Brain Sodium Channels

Kristopher M. Kahlig, Ryoko Hirakawa, Lynda Liu, Alfred L. George, Jr., Luiz Belardinelli and Sridharan Rajamani

Department of Biology (Cardiovascular Therapeutic Area) Gilead Sciences Inc., Foster City and Fremont, CA. (KMK, RH, LL, LB, SR)

Departments of Medicine and Pharmacology, Vanderbilt University School of Medicine, Nashville, TN. (AG)
Running Title Page

Running Title: Ranolazine reduces hippocampal neuronal excitability

Corresponding Author: Sridharan Rajamani
Department of Biology
Gilead Sciences
7601 Dumbarton Circle
Fremont, CA 94555
Phone: (510) 739-8454, Fax: (510) 739-8401
Sridharan.Rajamani@gilead.com

Number of Text Pages 23
Number of Figures 8
Number of Tables 3
Number of References 30

Abstract Word Count 250
Introduction Word Count 592
Discussion Word Count 1257

Nonstandard Abbreviations:
  RAN       ranolazine
  I_{Na}     Na⁺ current
  TTX       Tetrodotoxin
Abstract
Ranolazine is an approved drug for chronic stable angina that acts by suppressing a non-inactivating current conducted by the cardiac sodium channel (persistent $I_{\text{Na}}$). Ranolazine has also been shown to inhibit the increased persistent $I_{\text{Na}}$ carried by Na$_v$1.1 channels encoding epilepsy and migraine associated mutations. Here, we investigate the antiepileptic properties of ranolazine exhibited through the reduction of hippocampal neuronal excitability. At therapeutically relevant concentrations, ranolazine reduced action potential (AP) firing frequency of hippocampal neurons in response to repetitive depolarizing current injections. Similarly, using a single current injection paradigm, ranolazine required a long depolarization (4s) to produce significant inhibition of excitability, which was similar to that observed for the anticonvulsants phenytoin (slowly binds to the fast-inactivated state) and lacosamide (binds to the slow-inactivated state). Ranolazine enhanced the development of fast and slow inactivation assessed with conditioning pre-pulses of 100, 1000 or 10000ms. Recovery of channels from inactivated states was also slowed in the presence of ranolazine. Interestingly, the use-dependent inhibition of hippocampal neurons was dependent on the duration of the voltage step suggesting ranolazine does not selectively affect the open state and may also interact with inactivated states. NEURON computational simulations predict equal inhibition of AP generation for binding to either fast inactivated or slow inactivated states. Binding of ranolazine to either pre-open or open states did not affect the excitability of the simulation. Ranolazine was able to significantly reduce the epileptiform activity of the neuronal cultures suggesting possible antiepileptic activity.
**Introduction**

Epilepsy is a common neurologic disorder characterized by abnormal neuronal synchronization (Rogawski and Loscher, 2004). In several epileptic syndromes, the local network synchrony spreads to include one or both hemispheres in a process termed secondary generalization. In the most severe form generalization produces a life-threatening, non-terminating seizure (generalized tonic-clonic seizure). Current antiepileptic therapeutics targeting neuronal voltage-gated sodium (NaV) channels selectively reduce Na⁺ current (I₉) during periods of high frequency firing (Rogawski and Loscher, 2004). Drug binding is thought to stabilize the Na⁺ channel inactivation process, which serves to dampen neuronal activity and network synchronization, while leaving normal neuronal functioning largely intact. CNS voltage-gated Na⁺ channels are responsible for the generation and propagation of neuronal action potentials. Several genetic epilepsies are caused by mutation of sodium channel genes SCN1A and SCN2A, which encode the pore forming α-subunits of the NaV1.1 and NaV1.2 isoforms, respectively (Catterall et al., 2008).

Ranolazine is an anti-anginal drug that has been shown to inhibit cardiac persistent I₉ at a therapeutic concentration of 2-8 μM (Antzelevitch et al., 2004; Chaitman, 2006). Furthermore, ranolazine has been shown to preferentially block the persistent I₉ evoked by mutations in Naᵥ1.5 (Fredj et al., 2006; Rajamani et al., 2009) as well as toxin or mutation induced persistent I₉ carried by muscle (Naᵥ1.4 (Wang et al., 2008)) and peripheral nerve (Naᵥ1.7 and Naᵥ1.8 (Rajamani et al., 2008; Wang et al., 2008)) Na⁺ channels. More recently, it was demonstrated that at an achievable brain concentration (~1μM), ranolazine blocked the increased ramp and persistent I₉ carried by epilepsy and migraine associated Naᵥ1.1 mutations more potently than the observed inhibition of transient (peak) I₉ (Kahlig et al., 2010a). However the ability of ranolazine to exert antiepileptic actions has not been reported.
Neuronal network excitability is achieved through a balance between transient (peak) $I_{Na}$ and persistent $I_{Na}$. Transient $I_{Na}$ generates the upstroke of the action potential and supports signal propagation. Although elevated levels of persistent $I_{Na}$ are associated with disease causing Na+ channel mutations, recent work has confirmed that a small fraction of total $I_{Na}$ is normally conducted through a persistent mechanism. This depolarizing persistent $I_{Na}$ facilitates the integration of synaptic inputs and supports repetitive firing capabilities. Moreover, the normal balance between transient and persistent $I_{Na}$ is activity-dependent with periods of high stimulation frequency (e.g. seizure) increasing the proportion of persistent $I_{Na}$.

The consensus mechanism of action of typical Na+ channel targeting antiepileptic drugs, such as phenytoin, has been the reduction of high frequency firing by the inhibition of transient $I_{Na}$. In 1994 Kuo and Bean refined this mechanism by demonstrating phenytoin inhibition of hippocampal Na+ channels by the slow binding to fast inactivated conformations (Kuo and Bean, 1994). Studies by other investigators have shown that phenytoin can also reduce the persistent $I_{Na}$ normally expressed by several neuron types (Chao and Alzheimer, 1995; Lampl et al., 1998) (therapeutic concentration 4-8 µM (Richens, 1979; Sherwin et al., 1973)). However, the contribution of persistent $I_{Na}$ to seizure and antiepileptic activity remains to be clarified.

Lacosamide is a novel antiepileptic drug that selectively stabilizes slow inactivated states of brain NaV channels with minimal interactions with fast inactivated conformations (therapeutic concentration 17-41 µM (Ben-Menachem et al., 2007)). Previous reports have demonstrated an inhibition of neuronal excitability with corresponding reduction of transient $I_{Na}$. The independent mechanisms of action of phenytoin and lacosamide provide useful tools to investigate the effects of ranolazine on brain neuronal excitability.

In this study, we investigated the antiepileptic properties of ranolazine using hippocampal neuronal cultures. Ranolazine reduced the number and frequency of action potentials evoked by depolarizing current injections with a time course similar to phenytoin and lacosamide. Computational modeling showed that this inhibition of neuronal NaV channels by
ranolazine can result from either binding to fast-inactivated or slow-inactivated states with minimal interactions with other channel conformations. In addition, ranolazine decreased the epileptiform activity induced by NMDA activation (removal of extracellular Mg$^{2+}$). Our findings provide an initial observation that suggests ranolazine could be effective in controlling high frequency firing during epileptic seizure.
**Materials and Methods**

**Primary Neuronal Culture**

All animal procedures were performed in strict adherence to the policies and procedures approved by the Gilead Sciences IACUC. Rat hippocampal neurons were cultured as previously described with a slight modification (Brewer et al., 1993). Briefly, hippocampi were isolated from brains of newborn Sprague-Dawley rats (postnatal day 1-3, sex undetermined), followed by dissociation using papain (1mg/mL; Worthington Biochemical, Lakewood, NJ, USA) and gentle trituration. Cells were diluted in primary cell culture medium (Neurobasal-A medium (Invitrogen, Carlsbad, CA, USA), 2% B27 supplement (Invitrogen), 0.5mM L-glutamine, 100U/mL penicillin and 100mg/ml streptomycin) at a density of 6.5 x 10^5 cells/ml. A volume of 1 mL was added to a 35mm dish containing coverslips coated with poly-D-lysine and laminin (BD Biosciences, Sparks, MD, USA), which resulted in approximately 700 cell/mm². 48 hours after seeding, the media was changed and cytosine β-D-arabinoside (10µM) was added to prevent the proliferation of non-neuronal cells. Subsequently, media was changed every 3-5 days. Neurons were cultured for at least 10 days prior to experimentation. Neurons exhibiting pyramidal morphology were used for these studies. Unless otherwise noted, all reagents were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.).

**Stably Expressed hNa\textsubscript{v}1.2**

HEK cells stably expressing the human brain Na\textsubscript{v}1.2 channel were described previously (Wang et al., 2010). The cell line was created by simultaneous stable integration of piggyBac transposons encoding the cDNA for either SCN2A (G418 selection) or SCN1B-IRES2-SCN2B (puromycin selection), as described previously (Kahlig et al., 2010b). The cells were grow in DMEM High Glucose (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 2mM L-
glutamine, 100U/mL penicillin, 100ug/mL streptomycin, 1mg/mL G418 and 3ug/mL puromycin
(Invivogen, San Diego, CA, USA).

Electrophysiology

All experiments were performed at room temperature using a MultiClamp 700B amplifier,
Digidata 1400 digitizer and pClamp 10 software (Molecular Devices, Sunnyvale, CA, USA).
Patch electrodes (2-3MOhm) were fabricated from borosilicate glass capillary tubes (World
Precision Instruments, Sarasota, USA) using a DMZ-Universal Puller (Zeitz Instruments GmbH,
Martinsried, Germany). Results are presented as mean ± SEM. Unless otherwise noted, data
were analyzed with GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA) and
statistical comparisons were made using one-way ANOVA followed by a Tukey post-hoc test in
reference to the control drug free condition. Steady-state channel availability curves were fit with
Boltzmann functions to determine the voltage for half-maximal activation/ inactivation (V1/2) and
a slope factor (k). Recovery from inactivation was evaluated by fitting the peak current recovery
with a two exponential function, \( \frac{I}{I_{\text{max}}} = A_f \times [1 \times \exp(t/\tau_f)] + A_s \times [1 \times \exp(t/\tau_s)] \), where \( \tau_f \) and \( \tau_s \)
denote time constants (fast and slow components, respectively), \( A_f \) and \( A_s \) represent the fast
and slow fractional amplitudes. For use-dependence studies, currents in response to pulse 40
(\( P_{40} \)) were normalized to the peak current recorded in response to the first pulse in each
frequency train.

Whole-cell Voltage-Clamp

Whole-cell voltage-clamp recordings were performed as described previously (Kahlig et al.,
2010a). For voltage-clamp experiments with neuronal cultures, the pipette solution consisted of
(in mM) 140 CsCl, 2 MgCl₂, 1 EGTA, 10 HEPES, with a pH of 7.3 and osmolarity of
310mOsmol/kg. The bath solution contained in (mM): 140 NaCl, 2.5 KCl, 1 CaCl₂, 1 MgCl₂, 10
dextrose, 10 HEPES, with a pH of 7.4 and osmolarity of 290mOsmol/kg. For voltage-clamp
experiments with stably expressed hNa\(_{\text{V}1.2}\) in HEK cells, the pipette solution consisted of (in mM) 110 CsF, 10 NaF, 20 CsCl, 2 EGTA, 10 HEPES, with a pH of 7.35 and osmolarity of 300 mOsmol/kg. The bath solution contained in (mM): 145 NaCl, 4 KCl, 1.8 CaCl\(_2\), 1 MgCl\(_2\), 10 dextrose, 10 HEPES, with a pH of 7.35 and osmolarity of 310 mOsmol/kg. For all solutions, the osmolarity was adjusted with sucrose. Cells were allowed to stabilize (3 min for neurons and 10 min for stably expressed Na\(_{\text{V}1.2}\)) after establishment of the whole-cell configuration before current was measured. For all voltage-clamp experiments, series resistance was compensated 90% to minimize voltage error. Leak currents were subtracted by using an online P/4 procedure and all currents were low-pass Bessel filtered at 4 kHz and digitized at 50 kHz. Specific voltage-clamp protocols were used as depicted in figure insets.

Whole-cell Current-Clamp

Whole-cell current-clamp recordings were performed as described previously (Mitterdorfer and Bean, 2002). The pipette solution consisted of (in mM) 140 K-gluconate, 2 MgCl\(_2\), 10 EGTA, 10 HEPES, 0.5 Mg-ATP with a pH of 7.3 and osmolarity of 300 mOsmol/kg. The bath solution contained in (mM): 140 NaCl, 5 KCl, 2.5 CaCl\(_2\), 1 MgCl\(_2\), 10 dextrose, 10 HEPES, with a pH of 7.4 and osmolarity of 320 mOsmol/kg. For experiments investigating spontaneous activity in the culture (action potential generation and synaptic transmission), the MgCl\(_2\) in the bath solution was isosmotically replaced by sucrose. Cells were allowed to stabilize for 3 min after establishment of the whole-cell configuration before switching to current-clamp mode. For all experiments, pipette neutralization and bridge balance corrections were utilized to minimize voltage errors and pipette filtering. All voltage records were low-pass Bessel filtered at 2 kHz and digitized at 10 kHz. For experiments investigating evoked action potential generation, the neuron was stimulated for either 1 or 4 s using a depolarizing current injection of 1.5X threshold. Only neurons with a resting membrane potential more negative than -50 mV were used in this study and the resting membrane potential of the neuron was not modified. Ranolazine (10 µM) did not
alter the resting membrane potential compared to the drug free condition (-64.0 ± 1.5 mV versus -64.4 ± 1.4 mV for control and ranolazine, n = 20 respectively).

In vitro pharmacology

A stock solution of 50mM ranolazine (Gilead Sciences, Foster City, CA) was prepared in 0.1M HCl. A fresh dilution of ranolazine in the bath solution was prepared every experimental day and the pH was readjusted as necessary. Direct application of the test solution to the clamped cell was achieved using the Perfusion Pencil system (Automate, Berkeley, CA). Direct cell superfusion was driven by gravity at a flow rate of 350μL/min using a 250micron tip. This system sequesters the cell within a stream and enables complete solution exchange within one second. The cell was superfused continuously starting immediately after establishing the whole-cell configuration. Control currents were measured in drug-free solution. Drug containing solutions were superfused for 1.5mins prior to current recordings to allow equilibration of tonic drug block (tonic block). Tonic block of peak current was measured from this steady-state condition using a depolarizing voltage step at a frequency of 0.2Hz. Use-dependent block of peak current was measured during pulse number 40 of a depolarizing pulse train at either 10 or 25Hz. Concentration inhibition curves were fit with the Hill equation: 

\[ \frac{I}{I_{\text{max}}} = \frac{1}{1 + 10^{(\log_{10}C_{50} - I) \cdot n_h}} \]

where \( C_{50} \) is the concentration that produces half inhibition and \( n_h \) is the Hill coefficient factor. The upper and lower limits were set to 1 and 0.

Computational Modeling

The computational model reported here for a brain Na\( _V \) channel is based on our previously described model of human brain Na\( V_1.1 \) (Kahlig et al., 2006). The model has been simplified and generalized to allow better quantititation of drug binding to fast inactivated and slow inactivated states (Figure 6). Figure 6A illustrates the Markov model, which includes states for
the conditions: closed (C), pre-open (PO), open (O), fast inactivated (FI) and slow inactivated (SI). Transitions between states are reversible and described by continuous equations whose instantaneous solution depends on the membrane voltage. Microscopic reversibility was ensured by setting the rate constant for $\beta_6$ equal to $(\beta_4 \alpha_6 \beta_5)/(\alpha_4 \alpha_5)$. Rate equations for all transitions are reported in Table 3. The state $O$ represents the only conducting state and the occupancy of $O$ determines the sodium current by: $I_{Na} = G_{Na\_bar} \cdot O \cdot (V - E_{Na})$, where $I_{Na}$ is the sodium current, $G_{Na\_bar}$ is the maximum sodium current density, $O$ is the fractional occupancy of the open state, $V$ is the membrane voltage and $E_{Na}$ is the sodium reversal potential.

To evaluate channel gating, NaV-Model was inserted into a single compartment model of length of 20.0 $\mu$m, diameter of 12.1 $\mu$m and membrane capacitance of 1 $\mu$F/cm$^2$. The resulting computational surface area is equivalent to the average surface area measured previously for HEK cells ($7.6 \times 10^{-6}$ cm$^2$, n=15) (Rhodes et al., 2005). Voltage protocols used to assess activation, steady-state fast inactivation, recovery from fast inactivation, entry into slow inactivation, voltage-dependence of slow inactivation and recovery from slow inactivation are included as figure insets. The reported model accurately reproduces all recorded behaviors of heterologously expressed brain NaV channels (Supplemental Figure 3) (Kahlig et al., 2006). For action potential simulations, we used the somatic compartment of the previously published model of a pyramidal neuron (Uebachs et al., 2010). Our NaV model (0.37S/cm$^2$) replaced all sodium channels in the original model. The potassium channel current densities were adjusted to account for the different behaviors between our Markov-style model and the original HH-style NaV models. The simulated values were (in S/cm$^2$) 0.002 $I_{KCT}$, 0.00025 $I_{KAHP}$, 0.0032 $I_{KM}$, 0.002 $I_{KA}$, 0.002 $I_{KDR}$, 0.00004 $I_{Kleak}$, 0.012 $I_{Kslow}$. The resting membrane potential was initialized at -75mV and the model was allowed 5000ms simulation time for parameter stabilization prior to depolarizing current injection.
Inhibition of peak $I_{\text{Na}}$ or action potential generation was simulated using four independent schemes (Figure 6D) to evaluate binding to the following states: pre-open ($PO$), open ($O$), fast inactivated ($FI$) or slow inactivated ($SI$). The apparent binding rates for ranolazine are $K_{\text{ON}} = 1 \text{M}^{-1} \text{ms}^{-1}$ and $K_{\text{OFF}} = 5 \times 10^{-5} \text{ms}^{-1}$ (Figure 6C, apparent $K_d$ of 50$\mu$M). The microscopic binding rates were estimated by $K_{\text{app}} = K_i / (1-h)$, where $K_{\text{app}}$ is the apparent dissociation constant for the inactivated state, $K_i$ is the dissociation constant for the inactivated state and $h$ is the fraction of available channels (Kuo and Bean, 1994). This relationship assumes minimal binding to closed states and accounts for reduced availability of the high affinity binding site due to membrane hyperpolarization. The apparent binding rates were measured at a holding potential of -70mV, which results in 66±6% channel availability (Figure 6C, $n=6$, $h=0.66$). The unbinding rate ($K_{\text{OFF}}$) was kept constant and the microscopic $K_{\text{ON}}$ was calculated as $2.9 \text{ M}^{-1} \text{ms}^{-1}$ ($0.99 \text{ M}^{-1} \text{ms}^{-1} / 0.34$) and simulations were performed using a microscopic $K_{\text{ON}}$ of 3 $\text{M}^{-1} \text{ms}^{-1}$ (microscopic $K_d \sim 16.67 \mu$M to the inactivated state). This 3-fold increase corresponds well with the 4.5-fold increase in the $K_{\text{ON}}$ (apparent to microscopic) measured for phenytoin (Kuo and Bean, 1994). Additional simulations were performed testing $K_{\text{ON}}$ values of 10 and 20$\text{M}^{-1}\text{ms}^{-1}$ (Kd values of 5 $\mu$M and 2.5 $\mu$M, respectively) in an attempt to force binding to $PO$ and $O$ states.

Computational modeling was performed using NEURON (www.neuron.yale.edu (Hines and Carnevale, 2001)). All simulations were performed using the default integration strategy (Backward Euler) with an implicit fixed time step of 25$\mu$s to maintain temporal accuracy and efficiency. Simulations were implemented on a Dell Precision T3500 employing a Quad Core Intel Xeon dual-core 3.2 GHz processors running Windows 7.
Results

Ranolazine inhibits evoked action potential generation

Figure 1A shows representative experiments in which cultured hippocampal neuronal action potentials were evoked in response to a 1s depolarizing current injection in the absence of drug (Figure 1A, top, CTR). Application of 10 μM ranolazine (RAN) had a minimal effect on the instantaneous firing frequency. However, the average number of action potentials generated was significantly decreased from 18.5±1.8 in CTR to 16.3±1.2 in the presence of RAN (Figure 1B, p<0.05). In separate experiments, the effect of ranolazine to reduce evoked action potentials was compared to phenytoin and lacosamide. Application of 3 μM phenytoin (Figures 1A, middle, DPH) or 30 μM lacosamide (Figures 1A, bottom, LCM) produced a similar inhibition pattern of evoked action potentials. This inhibition caused a significant reduction in average number of action potentials evoked in the presence of phenytoin (Figure 1B middle, 10.7±1.5 in CTR compared to 8.7±1.5 in the presence of phenytoin, p<0.05). The reduction did not reach significance for lacosamide treated neurons likely reflecting the delayed accumulation of slow inactivated states (Figure 1B, bottom).

Ranolazine (10 μM) was able to induce cessation of evoked action potential generation when the depolarizing pulse duration was 4s (Figure 2A, top). On average the number of action potentials generated during CTR was 49.2±3.3, compared to 28.0±4.9 in the presence of 10 μM ranolazine (Figure 2B, p<0.1). Similarly, for both phenytoin and lacosamide, extending the depolarizing injection duration to 4s resulted in firing cessation (Figure 2A) due to accumulated inhibition of Na\textsubscript{v} activity. The number of action potentials generated during CTR was 46.3 ± 3.5, compared to 24.7 ± 5.9 in presence of 3 μM phenytoin (Figure 2B, p<0.05). For experiments with lacosamide, the number of action potentials generated during CTR was 56.0 ± 5.5, compared to 44.3 ± 5.4 in the presence of drug (Figure 2B, p<0.05).
The effect of ranolazine on action potential firing was next assessed during nine sequential depolarizing current injections (1s, 0.667Hz). Figure 3A shows representative evoked action potential trains measured during pulses 1 and 9 in the absence of drug (CTR) or following sequential superfusion of either 3 μM or 10 μM ranolazine. The accumulation of Na\text{V} inhibition in the presence of 10 μM RAN was sufficient to cause firing cessation (denoted by an arrow).

Figure 3B shows the slow kinetics of inhibition as evidenced by a gradual reduction in the instantaneous firing frequency compared to CTR for each pulse. The average number of action potentials evoked for each pulse was plotted in Figure 3C. The inhibition increased during the pulse train suggesting a slowly developing block of Na\text{V} channels similar to that previously described for phenytoin and lacosamide (Errington et al., 2008). Compared to control, the average number of evoked action potentials during pulse 9 was significantly reduced for 10 μM ranolazine (20.3±3.0 to 7.5±1.4, respectively, p<0.05). These data suggest the slow kinetics of Na\text{V} block by ranolazine during an extended depolarizing pulse likely reflects either: (1) slow interaction of drug with a site that rapidly becomes available (fast inactivated states) or (2) rapid interaction with a site that slowly becomes available (slow inactivate states).

**Ranolazine modulates Na\text{V} fast inactivation**

We next determined the effect of 10 μM ranolazine on hippocampal I_{Na} during voltage protocols designed to selectively engage fast inactivation. Fast inactivation was induced using 100 ms depolarizing voltage steps to various potentials in the absence of drug (CTR) or following superfusion of 10 μM ranolazine (RAN) (Supplemental Figure 1). Ranolazine induced a negative shift in the V_{1/2} of steady-state inactivation (-5 mV, Table 1). The experiment was repeated with a longer (1000 ms) inactivating prepulse designed to allow additional time for ranolazine to interact with the channel (Supplemental Figure 2). The longer prepulse potentiated the shift in the V_{1/2} of steady-state inactivation (-8 mV, Table 1). There was a non-significant
trend toward delayed recovery of hippocampal Na\textsubscript{V} channels from fast inactivation in the presence of ranolazine (Supplemental Figure 1C, Table 1). Together, these data suggest ranolazine slowly stabilizes fast inactivation by interacting with Na\textsubscript{V} channel fast inactivated states.

Ranolazine modulates Na\textsubscript{V} slow inactivation

We next evaluated the effect of 10 \mu M ranolazine on hippocampal I\textsubscript{Na} during voltage protocols engaging both fast and slow inactivation. Inactivation was induced using long (10s) depolarizing voltage steps to various potentials in the absence of drug (CTR) or following superfusion of 10 \mu M ranolazine (RAN) (Supplemental Figure 1D). Ranolazine caused a significant negative shift in the V\textsubscript{1/2} of steady-state slow inactivation compared to CTR (Table 2). In addition, slow inactivation was more complete in the presence of ranolazine (residual availability 38 \pm 7\% for RAN compared to 55 \pm 6\% for CTR, p<0.001). Ranolazine also delayed the recovery of hippocampal Na\textsubscript{V} channels from slow inactivation induced by a maximally inactivating prepulse (Supplemental Figure 1E). The time constants of channel recovery were significantly larger in the presence of ranolazine without alterations to the amplitude of either the fast or slow recovery components (Table 2). These data suggest that ranolazine may interact with Na\textsubscript{V} slow inactivated states similar to the mechanisms of action proposed for lacosamide (Errington et al., 2008). However, a contribution of fast inactivated state binding cannot be excluded because these voltage protocols engage both fast and slow inactivation mechanisms.

Evaluation of tonic block and use-dependent block

Na\textsubscript{V} channel inhibitors typically exhibit minimal interaction with closed conformations, which can be assessed using hyperpolarizing holding potentials (tonic block, TB). The inhibition potency increases with rapid, repetitive stimulation (use-dependent block, UDB). Figure 4A shows the
minimal level of $I_{\text{Na}}$ tonic block (0.2 Hz, left) in a representative cell measured in the presence of 10 $\mu$M ranolazine (RAN) compared to the drug free condition (CTR). Increasing the stimulation frequency to 10 Hz or 25 Hz increased the level of inhibition. Figure 4B shows the levels of tonic block and UDB for increasing concentrations of ranolazine. The minimal inhibition observed for TB and 10 Hz preclude determination of the IC$_{50}$. Fitting the concentration-inhibition data for 25 Hz resulted in an estimated IC$_{50}$ of 114±83 $\mu$M (Hill coefficient = 0.6±0.1).

Previous reports investigating ranolazine block of non-brain Na$_V$ channel isoforms have found evidence of open or pre-open state inhibition using UDB voltage pulse trains with variable step durations (Rajamani et al., 2008; Wang et al., 2008; Zygmunt et al., 2011). Short voltage steps (2ms) increase the available sites for pre-open and open states by minimizing the presentation of inactivated conformations. Longer steps (20ms) would allow for additional inactivated states to become available. Equal potency for 2ms or 20ms step durations would suggest minimal effects of RAN on fast inactivated states. Thus, the potency of ranolazine UDB (25Hz, 40 pulses) in hippocampal neurons using voltage step trains of variable step duration (2ms or 20ms) was determined. Figure 4C plots current records from a representative neuron showing the increased potency of UDB for a step duration of 20ms (right) compared to 2ms (left). Figure 4D shows the average UDB at pulse 40 was significantly greater for a step duration of 20ms (13.0 ± 0.9%) than 2ms (1.8 ± 1.4%, p<0.001). These data suggest that ranolazine does not selectively interact with pre-open or open states of the Na$_V$ channels expressed in hippocampal neurons and Na$_V$ inactivated states contribute significantly to the observed UDB.

**Ranolazine suppresses epileptiform activity in hippocampal cultures**

To investigate the antiepileptic potential of ranolazine, we determined the effect of ranolazine on epileptiform activity evoked by NMDA receptor activation within hippocampal neuronal cultures. Figure 5A shows (CTR) a representative experiment in which Mg$^{2+}$ was removed from the bath
solution to activate NMDA-dependent hyperexcitability (Rogawski and Loscher, 2004) (CTR). Ranolazine (10 μM, RAN) was able to reduce action potential firing, which recovered following washout (WASH). The lower traces in panel A show representative epileptiform activity on an expanded timescale. Figure 5B shows the average reduction in the frequency of the epileptiform bursts by 10 μM ranolazine (0.45 ± 0.07Hz for RAN compared to 0.78± 0.08Hz for CTR, p<0.05). These data suggest ranolazine may be capable of suppressing neuronal hyperexcitability during a seizure.

Ranolazine does not alter miniature synaptic activity or non-NaV mechanisms

The reduction in hippocampal epileptiform activity observed for ranolazine may reflect inhibition of NMDA transmission, potentiation of GABA transmission or alterations in Kv channel activity. The effect of ranolazine to modulate brain NMDA, GABA and Kv channels was directly investigated using hippocampal neurons in culture. These experiments demonstrate that ranolazine exerts minimal/no effect on each of the systems (Supplemental Figure 2).

We next tested if the reduction of epileptiform activity by ranolazine required NaV inhibition. Tetrodotoxin (TTX, 0.3 μM) was used to block all NaV channels expressed by the hippocampal neurons and spontaneous miniature synaptic currents reflecting action potential independent synaptic transmission were measured at -70mV. Figure 5C illustrates representative miniature synaptic currents measured before (CTR), after superfusion with 10 μM ranolazine (RAN) or following washout (WASH). Ranolazine had no effect on either the frequency (Figure 5D) or amplitude (Figure 5E) of the miniature synaptic currents. This suggests that ranolazine has little direct effect on synaptic vesicle release machinery.

Markov model of brain NaV channels
To investigate the state dependent interaction of ranolazine with brain \( \text{Na}_V \) channels, we developed a Markov model consisting of three closed states \( (C_4, C_3, C_2) \), a pre-open state \( (PO) \), a fast inactivated state \( (FI) \), a slow inactivated state \( (SI) \) and an open state \( (O) \). The open state is the only conducting state and the rate constants connecting the states were optimized to reproduce the generalized behavior of h\( \text{Na}_V \)1.1 and h\( \text{Na}_V \)1.2 channels. Figure 6A shows a diagram describing the Markov model. The model generates rapidly activating and inactivating inward currents in response to 20ms voltage steps to between -80 and +60mV from a holding potential of -120mV. The behavior of the model was further validated using standard voltage protocols investigating fast activation, steady-state fast inactivation, recovery from fast inactivation, voltage-dependence of slow inactivation, development of slow inactivation and recovery from slow inactivation (Supplemental Figure 3). The blue data points in each figure represent data recorded from heterologously expressed \( \text{Na}_V \)1.1 and represent standard responses used to develop the model (Kahlig et al., 2006).

**Ranolazine interacts slowly with \( \text{Na}_V \) channels**

To quantitate the interaction between ranolazine and brain \( \text{Na}_V \) channels, we directly measured the apparent binding rates for ranolazine to h\( \text{Na}_V \)1.2. Figure 6B show the results of a representative experiment where the voltage was stepped to 0mV (5ms, 0.2Hz) from a holding potential of -70mV. The black bars denote rapid application of either 30 \( \mu \text{M} \) or 100 \( \mu \text{M} \) ranolazine (RAN). The inhibition (red lines) and recovery (blue lines) of peak \( I_{\text{Na}} \) were well fit with a single exponential equation, which estimates the time constant of apparent binding or unbinding, respectively. In separated experiments, the speed of superfusion solution exchange was confirmed using 1 \( \mu \text{M} \) TTX which yielded an inhibition time constant of 0.6 ± 0.1s (n=6). This was an order of magnitude faster than the average apparent binding rate measured for 100 \( \mu \text{M} \) ranolazine (7.9 ± 1.0s, n=9).
In Figure 6C we determined the apparent $K_{ON}$ and $K_{OFF}$ of ranolazine binding at -70mV and the inverse of the average inhibition time constants were plotted against ranolazine concentration. The inverse of the average recovery time constant was plotted as the drug free condition. A linear regression provided an apparent $K_{ON}$ (slope) of 0.99M$^{-1}$ms$^{-1}$ and a $K_{OFF}$ (y-intercept) of 5.5x10$^{-5}$ms$^{-1}$ for ranolazine binding and unbinding, respectively. The microscopic $K_{ON}$ was calculated as 2.9 M$^{-1}$ms$^{-1}$. Unless otherwise noted, simulation studies used a $K_{ON}$ of 3M$^{-1}$ms$^{-1}$ and $K_{OFF}$ of 5x10$^{-5}$ms$^{-1}$. With a Markov model it is possible to predict the effect of drug binding to individual states. Figure 6D shows the four schemes tested for ranolazine binding to brain NaV channels: Scheme 1, pre-open state binding; Scheme 2, open state binding; Scheme 3, fast inactivated state binding; Scheme 4, slow inactivated state binding. To simply the computational simulations, the binding Schemes do not allow transitions between drug bound conformations. The binding of ranolazine to the Markov model was validated using each binding scheme and the apparent or microscopic binding kinetics (Supplemental Figure 4). Restricting binding to PO or O states had minimal effect, while binding to either the FI or SI state caused a block of peak $I_{Na}$ that exhibited the appropriate magnitude and kinetics (Figure 6B).

Computational modeling of ranolazine effects on neuronal excitability

We constructed a cellular model based on the soma of a previously published representation of a hippocampal pyramidal neuron (Uebachs et al., 2010). Figure 7A shows the response of the model to a +160pA depolarizing current injection from a resting membrane potential of -77.6mV. In the absence of drug (No Drug) the model generated evoked action potentials during the entire 4s depolarization. Drug binding was simulated using the microscopic $K_{ON}$ and $K_{OFF}$ (3M$^{-1}$ms$^{-1}$ and of 5x10$^{-5}$ms$^{-1}$, respectively) and a concentration of 10 μM as in Figure 2. Restricting binding to PO or O states had minimal effect, while binding to either the FI or SI state caused firing cessation late in the depolarization.. Figure 7B shows the same experiment
performed with $K_{\text{ON}}$ increased to $10 \text{M}^{-1}\text{ms}^{-1}$ in attempt to force binding to $PO$ or $O$ states. Significant effects on excitability were only observed for binding to $FI$ or $SI$ states. Figure 7C shows the instantaneous frequency calculated between each action potential for the sweeps in panels A and B. Drug binding to either the $FI$ or $SI$ state progressively reduced the rate of action potential generation leading to firing cessation (arrows). The number of action potentials evoked during 4 seconds was plotted for each binding scheme (Figure 7D). Additional simulations were performed with $K_{\text{ON}}$ values of 1 (apparent $K_{\text{ON}}$), 6, or $20 \text{M}^{-1}\text{ms}^{-1}$. Even at the highest $K_{\text{ON}}$, binding to either the $PO$ or $O$ state did not affect the number of evoked action potentials. In contrast, binding to either the $FI$ or $SI$ state reduced the number of action potentials depending on the rate of binding, which determines the rate of inhibition accumulation.

We next simulated the repetitive current injection experiments from Figure 3 to investigate the binding scheme(s) capable of reproducing the inhibition profile observed with ranolazine. Figure 8 shows the action potentials evoked using a depolarizing current injection train (1sec, $+100\text{pA}$, $0.667\text{Hz}$) for pulses 1 and 9. In the drug free condition (No Drug) the model generated action potentials during each pulse and the number of action potentials is denoted at the end of the pulse. Drug binding was then simulated at a concentration of $3 \mu\text{M}$ using the microscopic $K_{\text{ON}}$ ($3 \text{M}^{-1}\text{ms}^{-1}$) and the binding schemes in Figure 6D. Binding to the $FI$ or $SI$ states reduced the instantaneous frequency of action potential generation at pulse 9 with a concomitant decrease in the number of evoked action potentials (Figure 8A). The accumulated binding to $FI$ or $SI$ states resulted in firing cessation at the end of pulse 9 (arrow). The simulations were repeated with a drug concentration of $10 \mu\text{M}$ as in Figure 3 and only binding to the $FI$ or $SI$ states reduced the excitability of the model (Figure 8B). At this higher concentration, both binding schemes produced firing cessation during pulse 9 (arrows). Binding to the $PO$ or $O$ states had no effect on the number of evoked action potentials, even at this higher concentration. These data reproduce well the empirical data reported in Figure 3 and further
suggest that inhibition of hippocampal neurons by ranolazine can be reproduced by simulated binding to either fast inactivated or slow inactivated states.
Discussion

In this report, hippocampal neurons were used to investigate the effect of ranolazine on central neuron excitability. Ranolazine reduced the excitability of hippocampal neuronal cultures with a slow time course, which was similar to phenytoin and lacosamide. Moreover, ranolazine reduced the epileptiform activity induced by removal of extracellular Mg\(^{2+}\). Both experimental and computer simulations predict that the inhibition of \(i_{\text{Na}}\) could result from fast and/or slow inactivation state binding, which contrasts to the predicted open state binding of ranolazine proposed for other non-brain \(\text{Na}_\text{v}\) channel isoforms, including the cardiac \(\text{Na}_\text{v}1.5\) (Nesterenko et al., 2011; Wang et al., 2008).

\(\text{Na}_\text{v}\) channels are common targets for antiepileptic drugs due to their role in the initiation and propagation of action potentials in most excitable tissues (George, 2005). Phenytoin and lacosamide are antiepileptic drugs with well characterized actions on \(\text{Na}_\text{v}\) channels (Errington et al., 2008; Kuo and Bean, 1994). Kuo and Bean reported that phenytoin selectively binds to the fast inactivated state with slow binding rates (\(K_{\text{ON}}\) of \(\sim 10 \text{ M}^{-1}\text{ms}^{-1}\) and \(K_{\text{OFF}}\) ~6x10\(^{-5}\)ms\(^{-1}\)) and with minimal interactions to other states (Kuo and Bean, 1994). In contrast, lacosamide was shown to selectively bind to slow inactivated conformations with little or no binding to other conformations including fast inactivated states (Errington et al., 2008). Although differing mechanistically, phenytoin and lacosamide each suppressed hippocampal action potentials firing with a similar time course to that of ranolazine (Figures 1 and 2). These results implicate both fast and/or slow inactivated states as potential targets for ranolazine binding. In fact, both inactivation processes (fast and slow) were enhanced in the presence of ranolazine (Supplemental Figure 1). Ranolazine progressively potentiated entry into inactivation as the inactivating prepulse was extended from 100 to 10,000ms. This time course correlates well with the slow inhibition profile of ranolazine to \(\text{hNa}_\text{v}1.2\) (Figure 6). The recovery of hippocampal \(\text{Na}_\text{v}\) channels from slow inactivation was delayed in the presence of ranolazine suggesting the rate limiting transition is drug dissociation. In the drug free condition, the voltage protocols employed
selectively assess the independent kinetic processes termed fast inactivation and slow inactivation. However, the sequential presentation of open, fast inactivated and slow inactivated states makes definitive determination of the binding site of ranolazine impossible using only this approach. However, we can conclude that ranolazine exhibits minimal interaction with closed conformations of brain NaV channels as evidenced by a low level of tonic block observed at a holding potential of -100mV (Figure 4 and (Kahlig et al., 2010a)).

A common feature of NaV targeting drugs is an increase in potency with repetitive stimulation, which is thought to reflect either 1) altered presentation of or 2) differential interaction with the binding site(s) during the repetitive activation/inactivating gating cycle (Hille, 1977; Starmer et al., 1987). Ranolazine exhibited UDB (at 25Hz) of hippocampal NaV channels with an IC$_{50}$ of 114±83 μM. Repetitive stimulation mimics the rapid neuronal firing associated with seizure and suggests ranolazine maybe effective during periods of high neuronal discharge. Previous reports have also inferred open state binding by using a modified UDB protocol in which the stimulation frequency is fixed and the voltage step duration is varied (Rajamani et al., 2008; Wang et al., 2008; Zygmunt et al., 2011). With this design, the time of open state presentation is fixed while the presentation of fast/slow inactivation states increases with step duration. In our experiments, the block of hippocampal neurons by ranolazine was more potent with a UDB step duration of 20ms (13.0 ± 0.9%) compared to 2ms (1.8 ± 1.4%) suggesting an interaction with inactivated states (Figure 4).

Minimal open state binding to brain NaV channels contrasts with our previous report which found no correlation between step duration and inhibition potency for the cardiac NaV1.5 (Zygmunt et al., 2011). Our previous work used a maximum stimulation frequency of ~6.5Hz to drive ranolazine binding, which is a non-physiologically high rate for a human cardiac system. It is tempting to speculate that preferential open state binding to non-brain NaV isoforms results from differences in the binding site and/or lower firing rates compared to CNS neurons. Alternatively, this discrepancy may reflect the chosen experimental conditions as a more rapid
stimulation frequency (25Hz as in this study) may produce pulse duration dependent UDB. Moreover, our previous computational modeling efforts assumed that ranolazine requires a hydrophobic pathway to the binding site which is blocked by closure of the inactivation gate (Nesterenko et al., 2011). These mechanistic limitations were coded into the Markov model to the exclusion of inactivation state binding. This approach reproduces several features of the inhibition of NaV1.5 by ranolazine, including preferential binding to NaV1.5 during atrial versus ventricular cardiac action potential waveforms (Nesterenko et al., 2011). However, subsequent publication of the first crystal structure of a NaV channel revealed prominent and concentric hydrophobic pathways surrounding the channel’s conduction pathway through intra-membrane fenestrations (Payandeh et al., 2011). These new structural data support work from Hille in 1977 which found both hydrophilic and hydrophobic pathways for the interaction of NaV channels and local anesthetics (Hille, 1977). With insights from the crystal structure, further investigation will be required to determine if selective open state block of NaV1.5 is necessary as well as sufficient to describe ranolazine’s actions.

The data presented in this report support inactivated state binding for ranolazine to brain NaV channels. Targeted binding to inactivated states is a common theme of antiepileptic drugs and this approach is predicted to maintain normal neuronal responsiveness to incoming stimuli by sparing resting and open conformations (Rogawski and Loscher, 2004). Computational modeling of a hippocampal neuron (Uebachs et al., 2010) was used to explore the NaV binding sites that were sufficient to reproduce the inhibition of evoked action potentials observed by ranolazine. A simplified Markov model of a brain NaV channel was developed that accurately reproduced the behavior of a NaV channel and was based on our previously reported model of NaV1.1 (Kahlig et al., 2006). Simulated binding of ranolazine to either PO or O states had minimal effect on evoked action potentials even at a KON 20-fold greater than the measured apparent KON (1M⁻¹ms⁻¹ at -70mV, Figure 6). This lack of effect could reflect an underestimation of the true KON at physiologic temperatures and potentials. In fact the binding rate for phenytoin
to hippocampal neurons is ~3M⁻¹ms⁻¹ at -70mV but increases to ~5M⁻¹ms⁻¹ at -50mV and ~14M⁻¹ms⁻¹ at +40mV (Kuo and Bean, 1994). However, the microscopic K_ON calculated for ranolazine is 3M⁻¹ms⁻¹ and simulated binding of ranolazine to either FI or SI states using this microscopic rate produced a robust reduction in simulated action potentials (Figure 7 and 8) in parallel to that observed using hippocampal neuronal cultures (Figure 2 and 3). In addition, the binding rates used in our simulations compare well with those previously measured for carbamazepine (Kuo et al., 1997) (K_ON of 38M⁻¹ms⁻¹ and K_OFF of 6x10⁻⁴ms⁻¹) and lamotrigine (Kuo and Lu, 1997) (K_ON of 10M⁻¹ms⁻¹ and K_OFF of 8x10⁻⁵ms⁻¹). Therefore, it is likely that the inhibition exerted by ranolazine simply reflects binding to inactivated states of neuronal Na⁺ channels. The slow inhibition time course of ranolazine may reflect binding to either fast inactivated or slow inactivated states because the reduction of action potential generation was equally potent for simulated FI or SI binding.

A common experimental model of seizure is the induction of epileptiform activity by removal of extracellular Mg²⁺, a maneuver which activates excitatory NMDA ion channels (Rogawski and Loscher, 2004). In this model, ranolazine was able to reduce the epileptiform activity generated in the hippocampal neuronal cultures (Figure 5). The molecular target of this novel antiepileptic action is likely brain Na⁺ channels because ranolazine had no effect on the miniature synaptic potentials (Figure 5), GABA or NMDA neurotransmission or Kᵥ channels (Supplemental Figure 2).

Ranolazine does not exhibit Naᵥ channel isoform selectivity. This feature has been leveraged by previous in-vitro studies demonstrating ranolazine can normalize the excessive Naᵥ channel and/or neuronal activity underlying an array of clinical conditions, such as neuropathic pain, paramyotonia congenita, migraine and epilepsy (El-Bizri et al., 2011; Estacion et al., 2010; Hirakawa et al., 2012; Kahlig et al., 2010a). Additional studies, including animal models of epilepsy, are necessary to determine if the results presented here will translate into a therapeutic benefit.
Acknowledgments

The authors would like to acknowledge Steven Nguyen for his valuable technical assistance.
Authorship Contributions

Participated in Research Design – KK, RH, SR

Conducted Experiments – KK, RH, LL

Performed Data Analysis – KK, RH

Wrote or Contributed to the Writing of the Manuscript – KK, RH, AG, LB, SR
References


Rajamani S, Shryock JC and Belardinelli L (2008) Block of tetrodotoxin-sensitive, Na(V)1.7 and tetrodotoxin-resistant, Na(V)1.8, Na+ channels by ranolazine. *Channels (Austin)* **2**(6): 449-460.


Footnotes

KMK and RH contributed equally. This study was supported by Gilead Sciences.

Reprint requests should be directed to:
Sridharan Rajamani
Department of Biology
Gilead Sciences
7601 Dumbarton Circle
Fremont, CA 94555
Phone: (650) 739-8454, Fax: (510) 739-8401
Sridharan.Rajamani@gilead.com
Figure Legends

Figure 1. Ranolazine reduces central neuron excitability.

Hippocampal neuronal cultures were used to determine the effect of ranolazine on central neuron excitability. (A) Representative experiments showing action potentials evoked using depolarizing current injections (1s) performed either before (CTR) or after the application of 10 μM ranolazine (RAN), 3 μM phenytoin (DPH) or 30 μM lacosamide (LCM). The average resting membrane potentials in the absence of drug were -62.0 ± 1.5 mV, -63.1 ± 2.1 mV, and -61.0 ± 6.6 mV for the RAN, DPH and LCM recordings, respectively. (B) The average number of action potentials evoked during ranolazine, phenytoin and lacosamide treatments (n= 6, 3 and 3, respectively). Significant differences from the CTR condition are denoted by * = p<0.05.

Figure 2. Ranolazine induces cessation of action potential firing during the extended depolarization.

The experiments in Figure 1 were repeated using a longer 4s depolarizing current injection. (A) Representative experiments showing action potentials evoked from cultured hippocampal neurons using depolarizing current injections (4s) performed either before (CTR) or after the application of 10 μM ranolazine (RAN), 3 μM phenytoin (DPH) or 30 μM lacosamide (LCM). Representative experiments illustrating the cessation (arrow) of excitability observed during each drug treatment. The resting membrane potentials in the absence of drug were -65.1 ± 3.2 mV, -66.6 ± 5.1 mV, and -64.5 ± 6.0 mV for the RAN, DPH and LCM recordings, respectively. (B) All three drugs significantly reduced the average number of evoked action potentials during the extended depolarization (n= 5, 3 and 3, respectively). Significant differences from the CTR condition are denoted by * = p<0.05 and ** = p<0.01.
Figure 3. Repetitive depolarization potentiates the inhibition of neuronal excitability by ranolazine.

(A) Representative experiments illustrating action potentials evoked from cultured hippocampal neurons using a repetitive depolarizing current injection (1s, 0.67Hz) either before (CTR, top) or after the application of 3 μM ranolazine (middle) or 10 μM ranolazine (bottom). For clarity only data from Pulse 1 and Pulse 9 are shown and the arrows denote cessation of action potential generation. The resting membrane potential in the absence of drug was -70.0 ± 3.4 mV. (B) The instantaneous frequency plot of each action potential train in panel A illustrates the concentration and stimulation dependence of the reduction in excitability. Odd numbered data points are omitted for clarity. (C) Ranolazine (RAN, 10 μM) reduced the average number of action potentials evoked in response to pulse 9 demonstrating a delayed inhibition of neuronal excitability (n=4). Significant differences from the CTR condition are denoted by ** = p<0.01.

Figure 4. Ranolazine use-dependent block is modulated by frequency and step duration.

(A) Representative current traces illustrating tonic block (0.2Hz) or use-dependent block 10Hz or 25Hz assessed at the end of a 40 pulse train. Hippocampal neuronal I_{Na} was measured before (CTR) or after the application of 10 μM ranolazine (RAN). (B) The average tonic block and use-dependent block measured for various ranolazine concentrations. (C) Representative experiment showing the use-dependent inhibition of NaV currents by 10 μM ranolazine (RAN) in response to step durations of either 2ms (left) or 20ms (right). Note the increased inhibition at pulse 40 (P_{40}) for a step duration of 20ms compared to 2ms. (D) The average normalized peak current for each step in a 25Hz train plotted for either a 2ms (squares) and 20ms (triangles) step duration (n=5). For clarity, after step 5 only even numbered data points are shown. A statistically
significant reduction in channel availability was observed for the 20ms duration step for pulses
2-40, * = p<0.05. Dotted lines in A and C represent zero current.

Figure 5. Ranolazine inhibits epileptiform activity by acting extra-synaptically.

(A) Spontaneous epileptiform activity was induced in the hippocampal neuronal culture by
NMDA receptor activation with low extracellular Mg²⁺ (CTR). This epileptiform activity was
reversibly inhibited by application of 10 μM ranolazine (RAN). Lower panels show representative
epileptiform bursts on an expanded time scale. The resting membrane potential in the absence
of drug was -60.4 ± 1.7 mV. (B) Average of five experiments illustrating the statistically
significant reduction of epileptiform activity (*= p<0.05). (C-E) Miniature synaptic currents were
isolated using 0.3 μM TTX to block action potential generation. (C) Representative experiment
showing the application of 10 μM ranolazine (RAN) did not affect spontaneous miniature
synaptic currents. Ranolazine had no effect on either the frequency (D) or amplitude (E) of the
events (n=7) demonstrating minimal interaction with either pre- or post-synaptic targets.

Figure 6. Brain Naᵥ computational model and simulation of ranolazine binding.

(A) Inset, cartoon of a Markov model for brain Naᵥ channels developed to examine ranolazine
binding. Top, the model generates rapidly activating and inactivating inward current similar to
native and heterologously expressed Naᵥ channels. (B) Apparent binding kinetics for ranolazine
(RAN) were measured using hNaᵥ1.2 and step depolarizations (0mV, 5ms, 1Hz) from a holding
potential of -70mV. Fast application of either 30 μM or 100 μM ranolazine (black bars) resulted
in a rapid reduction in the peak current that was reversible upon return to drug free superfusion.
The red and blue lines are single exponential fits of either the binding or disassociation of
ranolazine, respectively. Kᵦᵦ (≈5x10⁻⁵ ms⁻¹) was calculated from the inverse of the average
disassociation rate constants (tau). (C) The inverse of the binding rate constants were plotted
against the concentration of ranolazine and the apparent $K_{ON}$ ($\sim1\text{M}^{-1}\text{ms}^{-1}$) was calculated as the slope of the best fit line (n=4-9 for each point). (D) Four schemes were used to probe the binding site(s) of ranolazine to the Markov model. Binding to the pre-open state ($PO$, Scheme 1), open state ($O$, Scheme 2), fast inactivated state ($FI$, Scheme 3) or slow inactivated state ($SI$, Scheme 4).

**Figure 7. Simulated ranolazine binding to $Na_v$ inactivated states is required to reduce neuronal excitability.**

Action potential simulations were performed to investigate the mechanism of delayed inhibition of neuronal excitability by ranolazine. (A and B) Depolarizing current pulses (4s) resulted in a train of evoked action potentials in the drug free condition (No Drug, top trace). Ranolazine binding was investigated using the microscopic binding rate constant ($K_{ON}$) of $3\text{M}^{-1}\text{ms}^{-1}$ (left) or an elevated $K_{ON}$ of $10\text{M}^{-1}\text{ms}^{-1}$ in an attempt to force binding to $PO$ or $O$ states (right). Note cessation of firing for binding to either $FI$ or $SI$ states (dark blue and light blue arrows, respectively) with minimal effect of simulated binding to either $PO$ or $O$ states. (C) Instantaneous frequency calculated for each condition in panels A (left) and B (right) illustrating a slowing in firing rate prior to spike cessation. (D) Number of action potentials evoked during the four seconds of depolarizing current injection. Binding targeting the $FI$ or $SI$ state reduced neuronal excitability in a $K_{ON}$ dependent manner, while binding to $PO$ or $O$ states had no effect. In A and B, scale bars represent 40mV and 1 s.

**Figure 8. Ranolazine induces cessation of stimulated evoked action potential during the repetitive depolarization.**

Repetitive pulses of depolarizing current (1s, 0.67Hz) evoked trains of action potentials in the hippocampal computational model (No Drug, top). The number of evoked action potentials is shown at the end of each trace. Ranolazine binding was investigated using the microscopic $K_{ON}$
(3M⁻¹ms⁻¹) and a drug concentration of either (A) 3 μM or (B) 10 μM. The top panels show the evoked action potentials generated during Pulse 1 (left) or Pulse 9 (right). The bottom panels illustrate the instantaneous frequency calculated during Pulse 1 (left) or Pulse 9 (right). The symbols for \( \text{PO} \) (red) or \( \text{O} \) (green) state binding are under the symbols denoting the drug free condition (black). Binding to either the \( \text{FI} \) or \( \text{SI} \) state resulted in a pulse dependent decrease in excitability reflected by a reduction in the number of evoked action potentials and firing cessation (dark blue and light blue arrows, respectively). Simulated drug binding to either the \( \text{PO} \) or \( \text{O} \) state had no impact on action potential number or firing frequency. The scale bars represent 40mV and 100ms.
Table 1. Biophysical parameters for fast inactivation

<table>
<thead>
<tr>
<th></th>
<th>Fast Inactivation (100ms)</th>
<th>Intermediate Inactivation (1000ms)</th>
<th>Recovery from Fast Inactivation $^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{1/2}$ (mV)</td>
<td>$V_{1/2}$ (mV)</td>
<td>$k$ (mV)</td>
<td>$\tau_f$ (ms) $\tau_s$ (ms) $n$</td>
</tr>
<tr>
<td>Control</td>
<td>-46.4 ± 1.3</td>
<td>5.1 ± 0.3</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>-51.0 ± 0.8</td>
<td>-4.7 ± 0.1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>6.5 ± 0.9 [80 ± 3%] 127 ± 15 [20 ± 3%] 5</td>
</tr>
<tr>
<td>Ranolazine (10μM)</td>
<td>-51.6 ± 2.0*</td>
<td>5.5 ± 0.2</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>-59.0 ± 1.7**</td>
<td>-5.1 ± 0.2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>8.1 ± 1.7 [73 ± 6%] 380 ± 211 [27 ± 6%] 5</td>
</tr>
</tbody>
</table>

$^6$ values in brackets represent fractional amplitudes. Values significantly different from Control are indicated as follows: *p<0.05, **p<0.01.
Table 2. Biophysical parameters for slow inactivation

<table>
<thead>
<tr>
<th></th>
<th>Slow Inactivation (10,000ms)</th>
<th>Recovery from Slow Inactivation §</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V_{1/2} (mV)</td>
<td>k (mV)</td>
</tr>
<tr>
<td>Control</td>
<td>-40.4 ± 2.1</td>
<td>-11.8 ± 0.7</td>
</tr>
<tr>
<td>Ranolazine (10μM)</td>
<td>-50.3 ± 3.0*</td>
<td>-11.5 ± 0.9</td>
</tr>
</tbody>
</table>

§ values in brackets represent fractional amplitudes. Values significantly different from Control are indicated as follows:

*p<0.05 and **p<0.001.
Table 3. Transition rates for NaV Markov model

<table>
<thead>
<tr>
<th>Transition</th>
<th>Forward Rate: $\alpha$ (ms$^{-1}$)</th>
<th>Reverse Rate: $\beta$ (ms$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) $C_4 \leftrightarrow C_3$</td>
<td>$55.23 \cdot \exp(v/16.39) + 0.4035$</td>
<td>$5.183 \cdot \exp(-v/68.35)$</td>
</tr>
<tr>
<td>(2) $C_3 \leftrightarrow C_2$</td>
<td>$54.4 \cdot \exp(v/16.8) + 0.2876$</td>
<td>$5.183 \cdot \exp(-v/68.35)$</td>
</tr>
<tr>
<td>(3) $C_2 \leftrightarrow PO$</td>
<td>$53.64 \cdot \exp(v/17.21) + 0.1709$</td>
<td>$5.183 \cdot \exp(-v/68.35)$</td>
</tr>
<tr>
<td>(4) $PO \leftrightarrow O$</td>
<td>$52.19 \cdot \exp(v/18.05) + 0.03247$</td>
<td>$0.04408 \cdot \exp(-v/18.54)+1.646$</td>
</tr>
<tr>
<td>(5) $O \leftrightarrow FI$</td>
<td>$0.5663 \cdot \exp(v/58.16) + 0.1467$</td>
<td>$0.001427 \cdot \exp(v/28.12)$</td>
</tr>
<tr>
<td>(6) $PO \leftrightarrow FI$</td>
<td>$3.128 \cdot \exp(-v/69.21)+1$</td>
<td>$\frac{(\beta_4 \cdot \alpha_6 \cdot \beta_5)}{(\alpha_4 \cdot \alpha_5)}$</td>
</tr>
<tr>
<td>(7) $FI \leftrightarrow SI$</td>
<td>$4.107 \cdot 10^4 \cdot \exp(v/355.8)+2.4 \cdot 10^5$</td>
<td>$6.3 \cdot 10^{-6} \cdot \exp(-v/22) + 3 \cdot 10^{-5}$</td>
</tr>
</tbody>
</table>

Drug Binding | Transition | Forward Rate: (ms$^{-1}$) | Reverse Rate: |
-------------|------------|--------------------------|--------------|
Scheme 1 | Pre-Open | $PO \leftrightarrow PO_B$ | $K_{ON} \cdot [Drug]$ | $K_{OFF}$ |
Scheme 2 | Open | $O \leftrightarrow O_B$ | $K_{ON} \cdot [Drug]$ | $K_{OFF}$ |
Scheme 3 | Fast Inactivated | $FI \leftrightarrow FI_B$ | $K_{ON} \cdot [Drug]$ | $K_{OFF}$ |
Scheme 4 | Slow Inactivated | $SI \leftrightarrow SI_B$ | $K_{ON} \cdot [Drug]$ | $K_{OFF}$ |

$K_{ON} = 1$ to $10M^{-1}ms^{-1}$ and $K_{OFF} = 5 \times 10^5ms^{-1}$
Figure 4

A

-100mV 0mV
20ms

0.2Hz 10Hz 25Hz

1nA

RAN CTR

2ms

B

Normalized Peak $I_{Na}$

RAN (µM)

0.2 Hz 10 Hz 25 Hz

C

-100mV 0mV
2ms 20ms 25Hz

CTR RAN

D

Normalized Peak $I_{Na}$

Pulse Number

2ms 20ms

CTR CTR RAN RAN
Figure 6

A. Model of Brain Na\textsubscript{v}.

B. Normalized peak \( I_{Na} \) for RAN concentrations of 30 \( \mu \text{M} \) and 100 \( \mu \text{M} \) over time (s).

C. \( 1/\tau \) as a function of RAN concentration (\( \mu \text{M} \)).

D. Simulated Drug Binding

<table>
<thead>
<tr>
<th>Scheme 1</th>
<th>Scheme 2</th>
<th>Scheme 3</th>
<th>Scheme 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Open State</td>
<td>Open State</td>
<td>Fast Inactivated State</td>
<td>Slow Inactivated State</td>
</tr>
<tr>
<td>( C_4 \leftrightarrow C_2 \leftrightarrow C_2 \leftrightarrow PO \leftrightarrow O )</td>
<td>( C_4 \leftrightarrow C_2 \leftrightarrow C_2 \leftrightarrow PO \leftrightarrow O )</td>
<td>( K_{On} \ast [\text{drug}] \uparrow K_{Off} )</td>
<td>( K_{On} \ast [\text{drug}] \uparrow K_{Off} )</td>
</tr>
<tr>
<td>( K_{On} \ast [\text{drug}] \uparrow K_{Off} )</td>
<td>( F_{I} \leftrightarrow S_{I} )</td>
<td>( F_{I} \leftrightarrow S_{I} )</td>
<td>( F_{I} \leftrightarrow S_{I} )</td>
</tr>
<tr>
<td>( PO_B )</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 7

A

\[ K_{ON} = 3 \text{M}^{-1}\text{ms}^{-1} \]

- No Drug
- Pre-Open (PO)
- Open (O)
- Fast Inactivated (FI)
- Slow Inactivated (SI)

B

\[ K_{ON} = 10 \text{M}^{-1}\text{ms}^{-1} \]

C

\[ K_{ON} = 3 \text{M}^{-1}\text{ms}^{-1} \]

\[ K_{ON} = 10 \text{M}^{-1}\text{ms}^{-1} \]

D

- No Drug
- Pre-Open
- Open
- Fast Inactivated
- Slow Inactivated

\[ K_{ON} (\text{M}^{-1}\text{ms}^{-1}) \]

- 1 (Apparent)
- 3 (Microscopic)
- 6
- 10
- 20