Multiple Modes of Ryanodine Receptor 2 Inhibition by Flecainide

D. Mehra, M. S. Imtiaz, D. F. van Helden, B. C. Knollmann, and D. R. Laver

School of Biomedical Sciences and Pharmacy, University of Newcastle and Hunter Medical Research Institute, Callaghan, New South Wales, Australia (D.M., M.S.I., D.F.v.H., D.R.L.); and Division of Clinical Pharmacology, Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee (B.C.K.)

Received July 7, 2014; accepted September 24, 2014

ABSTRACT

Catecholaminergic polymorphic ventricular tachycardia (CPVT) causes sudden cardiac death due to mutations in cardiac ryanodine receptors (RyR2), calsequestrin, or calmodulin. Flecainide, a class I antiarrhythmic drug, inhibits Na⁺ and RyR2 channels and prevents CPVT. The purpose of this study is to identify inhibitory mechanisms of flecainide on RyR2. RyR2 were isolated from sheep cardiomyocytes, and investigated by single-channel recording under various activating conditions, including the presence of cytoplasmic ATP (2 mM) and a range of cytoplasmic [Ca²⁺], [Mg²⁺], pH, and [caffeine]. Flecainide applied to either the cytoplasmic or luminal sides of the membrane inhibited RyR2 by two distinct modes: 1) a fast block consisting of brief substate and closed events with a mean duration of ~1 ms, and 2) a slow block consisting of closed events with a mean duration of ~1 second. Both inhibition modes were alleviated by increasing cytoplasmic pH from 7.4 to 9.5 but were unaffected by luminal pH. The slow block was potentiated in RyR2 channels that had relatively low open probability, whereas the fast block was unaffected by RyR2 activation. These results show that these two modes are independent mechanisms for RyR2 inhibition, both having a cytoplasmic site of action. The slow mode is a closed-channel block, whereas the fast mode blocks RyR2 in the open state. At diastolic cytoplasmic [Ca²⁺] (100 nM), flecainide possesses an additional inhibitory mechanism that reduces RyR2 burst duration. Hence, multiple modes of action underlie RyR2 inhibition by flecainide.

Introduction

In cardiac excitation-contraction coupling, the action potential depolarizes the L-type Ca²⁺ channel leading to Ca²⁺ release from the sarcoplasmic reticulum (SR) via ryanodine receptor 2 (RyR2) Ca²⁺ release channels located on the SR membrane (Nabauer et al., 1989). Following Ca²⁺ release, Ca²⁺ is sequestered into the SR via the SR Ca²⁺ ATPase or extruded from the cell by the Na/Ca exchanger (Dibb et al., 2007). The role of SR Ca²⁺ uptake and release (Ca²⁺ cycling) in maintaining the cardiac rhythm is highlighted by the arrhythmias associated with Ca²⁺ store overload. One such arrhythmia linked to the SR overload is catecholaminergic polymorphic ventricular tachycardia (CPVT) (Blayney and Lai, 2009). Mutations in RyR2 (Priori et al., 2001; George et al., 2003), calsequestrin (CASQ2) (Postma et al., 2002), or calmodulin (Nyegaard et al., 2012) can cause CPVT. These mutations increase RyR2 leak, causing spontaneous Ca²⁺ release due to excessive diastolic Ca²⁺ release. This activates the Na/Ca exchanger in the plasmalemma that produces the inward depolarizing current underlying the delayed after depolarizations leading to arrhythmias (Knollmann et al., 2006; Liu et al., 2006).

Flecainide is an orally administered, potent, antiarrhythmic agent that blocks cardiac sodium channels (Nav1.5) in a time- and voltage-dependent manner to reduce the maximum upstroke velocity of the action potential (Borchard and Boisien, 1982; Campbell and Vaughan Williams, 1983; Kojima et al., 1989). The kinetics of flecainide block of Nav1.5 have been extensively studied (Anno and Hondeghem, 1990; Nitta et al., 1992; Grant et al., 2000; Nagamoto et al., 2000; Liu et al., 2002). It has a relatively high affinity for Nav1.5 channels in their open and inactivated states (Grant et al., 2000; Liu et al., 2002) compared with their closed state. The recent discoveries that flecainide also blocked RyR2 channels, suppressed Ca²⁺ waves in CASQ2-/- cardiomyocytes, and prevented CPVT in mice and humans (Watanabe et al., 2009) suggest that RyR2 block may contribute to antiarrhythmic drug efficacy against Ca²⁺-triggered arrhythmias. This was demonstrated again more recently by the use of RyR2 block by carvedilol and its derivatives, which prevented stress-induced ventricular tachyarrhythmias in RyR2-mutant mice (Zhou et al., 2011). However, previous attempts to use RyR2 inhibitors to reverse effects of RyR2 mutations have not been successful in preventing arrhythmia (reviewed by McCauley and Wehrens, 2011; Watanabe and...
Knollmann, 2011). For example, the dual Na⁺ and RyR2 antagonist tetracaine did not suppress the Ca²⁺ waves in CASQ2⁻/⁻ myocytes upon prolonged exposure (Watanabe et al., 2009; Hilliard et al., 2010). Thus, it appears that it is not RyR2 block per se that is important in preventing Ca²⁺ overload arrhythmias. Therefore, there is a need to understand mechanisms for pharmacological inhibition of RyR2 by flecainide. Although the flecainide dose-response for RyR2 inhibition has been measured (Watanabe et al., 2009), no detailed study has been done to examine the action of this drug on the channel and how it depends on the RyR2 activation state.

Here, we use single-channel recording of RyR2 from sheep to develop a model for flecainide inhibition, which will provide an understanding of the action of the drug in cardiac muscle. The work reported here extends a previous finding that flecainide is an open-channel blocker (Hilliard et al., 2010). We now identify multiple flecainide inhibitory mechanisms that contribute to flecainide block of RyR2.

**Materials and Methods**

**Single-Channel Measurements.** SR vesicles containing RyR2 were isolated from sheep hearts and incorporated in artificial bilayer membranes as previously described (Laver et al., 1995). In brief, lipid bilayers were formed across the aperture of a delrin cup with a diameter of 150–250 mm using a lipid mixture of phosphatidyethanolamine and phosphatidylcholine (8:2 w/w; Avanti Polar Lipids, Alabaster, AL) in n-decane (50 mg/ml; ICN Biomedicals, Irvine, CA). During the SR vesicle fusion period, the cis (cytoplasmic) chamber contained 250 mM Ca⁺ (230 mM CaCl₂·2H₂O, 20 mM CaCl₂) + 1.0 mM CaCl₂, and the trans (luminal) chamber contained 50 mM Ca⁺ (30 mM CaCl₂·2H₂O, 20 mM CaCl₂) + 0.1 mM CaCl₂. When ion channels were detected in the bilayer, the trans Ca²⁺ was raised to 250 mM by aliquot addition of 4 M CaCl₂·2H₂O. During experiments, the composition of the cis solution was altered by a perfusion system, and the trans solution was altered by aliquot additions. The local perfusion (O’Neill et al., 2003) allowed exposure of single RyR2 to multiple drug concentrations applied in random sequence within ~3 seconds.

**Chemicals.** All solutions were pH buffered using 10 mM TES (N-tris[hydroxyethyl]methyl-2-aminoethanesulfonic acid) and titrated to pH 7.4 using CsOH (ICN Biomedicals). A Ca²⁺ electrode (Radiometer, Westlake, OH) was used in our experiments to determine the Ca²⁺ buffering and Ca²⁺ stock solutions as well as free [Ca²⁺] > 100 nM. Free Ca²⁺ was titrated with CaCl₂ and buffered using 4.5 mM BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; obtained from Invitrogen (Carlsbad, CA); free [Ca²⁺] < 1 μM) or dibromo-BAPTA (up to 2 μM; free [Ca²⁺] between 1 and 10 μM). Because all solutions applied in cis bath contained ATP (ATP chelates Ca²⁺ and Mg²⁺), free levels of Mg²⁺ (added as MgCl₂) were calculated using estimates of ATP purity and effective Mg²⁺ binding constants that were determined previously under our experimental conditions (Laver et al., 2004). The cesium salts were obtained from Sigma-Aldrich (St. Louis, MO). CaCl₂ and MgCl₂ were obtained from BDH Chemicals/VWR (Radnor, PA). Caffeine and flecainide were obtained from Sigma-Aldrich and were prepared as stock solutions in Milli-Q water (EMD Millipore, Billerica, MA).

**Data Acquisition and Analysis.** Experiments were carried out at room temperature (23 ± 2°C). Electric potentials were expressed using standard physiologic convention (i.e., current density relative to SR lumen at virtual ground). Control of the bilayer potential and recording of unitary currents was done using either an Axopatch 200B amplifier (Axon Instruments/Molecular Devices, Sunnyvale, CA) or a Bilayer Clamp-525C (Warner Instruments, Hamden, CT). Channel currents were digitized at 50 kHz and low-pass filtered at 5 kHz. Before analysis, the current signal was redigitized at 5–10 kHz and low-pass filtered at 1–3 kHz. Single-channel parameters, open probability, mean open time, and mean closed time were measured using a threshold discriminator at 50% of channel amplitude (Channel2 software by P. W. Gage and M. Smith, Australian National University, Canberra, Australia). Channel substate analysis was carried out using the hidden Markov model (Chung et al., 1990). The algorithm calculated the idealized, multilevel, current time course (i.e., background noise subtracted), and the transition probability matrix from the raw signal using maximum likelihood criteria.

Individual readings of open probability, mean open time, and closed times were derived from 30–60 seconds of RyR2 recording. Dwell-time histograms were compiled from 10⁶–10⁸ opening events. Hill equations were fitted to the dose-response data by the method of least squares. Average data are given as the mean ± S.E.M. The significance multigroup comparisons are made using analysis of variance (P < 0.01). The significance of the difference between pairs of control and test values was tested using Student’s t test (*P < 0.05 and **P < 0.01).

**Results**

**General Observations.** RyR2 were nearly maximally activated (open probability, Pₒ = 0.98 ± 0.02) by cytoplasmic Ca²⁺ (100 μM) in the presence of 2 mM ATP. Addition of flecainide to the cytoplasmic bath (membrane potential + 40 mV) induced two modes of RyR2 inhibition that were associated with channel closures on the millisecond and second timescales (fast and slow modes, respectively; Fig. 1A). The millisecond events represent transitions to a substate that are analyzed in detail later (Figs. 7 and 8). Our initial threshold analysis of channel gating lumped the substates together with complete channel closures.

Flecainide exhibited similar inhibiting kinetics when applied to the luminal side of the bilayer, albeit with a higher IC₅₀ (Fig. 1, B and C, cf. ○ cytosolic and ● luminal). The IC₅₀ for flecainide was strongly dependent on the ionic conditions (Fig. 1C; Table 1). Reducing the cytosolic ionic strength from 250 to 150 mM caused a 5-fold decrease in the IC₅₀ (Fig. 1C, cf. Δ and ○), decreasing cytosolic free [Ca²⁺] from 100 μM to 100 mM also caused a 5-fold decrease (cf. ○ and □), and adding 1 mM free Mg²⁺ to the cytosol caused a 3-fold decrease in the IC₅₀ (cf. ○ and △). The flecainide IC₅₀ values were also strongly dependent on membrane potential (Fig. 1D). IC₅₀ for cytosolic and luminal applications of flecainide had similar slopes on logarithmic plots of IC₅₀ versus bilayer voltage, decreasing by factors of 3.2 ± 0.2 (cytosolic; ○) and 3.6 ± 0.5 (luminal; ●) for each 25-mV depolarization. The Hill coefficients derived from fitting the data ranged from 0.6 to 2 (Table 1). The higher-than-unity values for some of the Hill coefficients indicate a multiple binding site mechanism for flecainide inhibition.

**Fast and Slow Modes of Flecainide Block at Systolic [Ca²⁺].** Since the potency of flecainide depended on the ionic conditions, we aimed to determine if this was a property of one or both modes of block. During Ca²⁺ release during systole, the [Ca²⁺] in the dyad cleft reaches 100 μM (Cannell and Soeller, 1997). In the presence of 100 μM cytoplasmic Ca²⁺ (2 mM ATP), addition of 50 μM flecainide to the cytosol induced fast inhibition with very few long closures (i.e., minimal slow block; Fig. 2A, middle trace), whereas long closures became much more apparent when 50 μM flecainide and 1 mM Mg²⁺ were added to the cytoplasmic bath (Fig. 2A, bottom trace). Mg²⁺ did not induce these long closures in the absence of flecainide (Fig. 2A, top trace). Addition of very high [Ca²⁺] (i.e., 0.4 and 1.6 mM) to the cytoplasmic bath in the absence of
Mg$^{2+}$ also increased the frequency of long channel closures (Fig. 2B). Thus, it appears that the potency of the slow mode of flecainide block is highly sensitive to the presence of divalent cations on the cytoplasmic side of the channel.

These effects of divalent ionic conditions on the kinetics of flecainide block were quantified by compiling frequency histograms of open and closed times from single-channel recordings (Fig. 2, C–E). Histograms were displayed using the log-bin method of Sigworth and Sine (1987), where individual exponential components appear as separate peaks centered on their time constant value. In these plots, the probability/frequency values are represented by their square roots because this provides uniform statistical scatter over the full range of times. Closed dwell-time histograms are presented as frequency of occurrence, $F_c(t)$, of closed durations ($t$) rather than their probability, $P_c(t) = P_c(t)/\tau_o$, where $\tau_o$ is the mean open time) to give a better indication of the total closing rates for each mode of block.

Open dwell-time probability histograms exhibited one or two exponential decays with an overall mean open duration of $\sim 100$ ms in the absence of flecainide. Flecainide applied to either the cytoplasmic (Fig. 2E) or luminal solutions (Supplemental Fig. 1) shifted the probability distributions to shorter times. The mean open duration, $\tau_o$, varied as the inverse of flecainide concentration, as shown in Fig. 2F.

RyR2 closed dwell-time frequency distributions exhibited two features in the absence of flecainide (with 1 mM cytoplasmic Mg$^{2+}$): an exponential decay with a time constant of 3 ms, which

### Table 1

Flecainide concentrations for half inhibition of RyR2 (IC$_{50}$), Hill coefficients ($H$), open probability in the absence of flecainide ($P_o$ max), and numbers of experiments ($n$) under various experimental conditions obtained from the dose-response curves in Fig. 1C. In each case, the luminal solution contained 250 mM Ca$^{2+}$, 100 $\mu$M Ca$^{2+}$, and no Mg$^{2+}$.

<table>
<thead>
<tr>
<th>Symbol (Fig. 1, C and D)</th>
<th>Side of Flecainide Addition</th>
<th>Cytoplasmic Ion Concentrations</th>
<th>IC$_{50}$</th>
<th>$H$</th>
<th>$P_o$ max</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ca$^+$</td>
<td>Ca$^{2+}$</td>
<td>Mg$^{2+}$</td>
<td>$\mu$M</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mM</td>
<td>$\mu$M</td>
<td>mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>○</td>
<td>cis</td>
<td>250</td>
<td>100</td>
<td>0</td>
<td>87 ± 6</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>●</td>
<td>trans</td>
<td>250</td>
<td>100</td>
<td>0</td>
<td>250 ± 50</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>△</td>
<td>cis</td>
<td>250</td>
<td>100</td>
<td>1</td>
<td>25 ± 4</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>◊</td>
<td>cis</td>
<td>150</td>
<td>100</td>
<td>1</td>
<td>5.0 ± 1.0</td>
<td>1.4 ± 0.8</td>
</tr>
<tr>
<td>□</td>
<td>cis</td>
<td>250</td>
<td>0.1</td>
<td>0</td>
<td>17 ± 2</td>
<td>2.0 ± 1.0</td>
</tr>
</tbody>
</table>
is characteristic of Mg$^{2+}$ inhibition (Fig. 2C, □; see arrow labeled $\tau_{Mg}$), plus the tail of a partially resolved peak with a submillisecond time constant. Addition of 50 $\mu$M cytoplasmic flecainide introduced two exponentials in the closed time distributions, with time constants of $\sim$1 ms (fast mode) and $\sim$1 second (slow mode), as seen in Fig. 2C (○, see arrows labeled $\tau_F$ and $\tau_S$, associated with fast and slow modes, respectively). In the absence of Mg$^{2+}$, (Fig. 2C, ●), the peak amplitude associated with the slow time constant was markedly reduced, whereas the peak associated with the fast time constant was relatively unaffected. A similar effect was seen when 500 $\mu$M flecainide was added to the luminal bath (Supplemental Fig. 1). Figure 2, B and D shows that increasing cytoplasmic Ca$^{2+}$ in the millimolar range had a similar effect on flecainide inhibition as Mg$^{2+}$. Thus, only the slow mode of flecainide block is sensitive to the divalent ionic conditions.

**Fast and Slow Modes of Flecainide Block at Diastolic [Ca$^{2+}$].** We also analyzed flecainide block at diastolic levels of cytoplasmic Ca$^{2+}$ (0.1 $\mu$M and 2 mM ATP) to see if it shared the same mechanisms as seen at systolic [Ca$^{2+}$] (Fig. 3). At these subactivating levels of Ca$^{2+}$, RyR2 had a $P_o = 0.14 \pm 0.04$ and mean open and closed times of $\sim$20 and $\sim$400 ms, respectively (Fig. 3A, top trace). Dwell time histograms of closed events (Fig. 3C, ○) exhibit two peaks centered at 0.2 and 200 ms. Addition of 50 $\mu$M flecainide to the cytoplasmic bath (Fig. 3A, bottom trace) 1) increased the long time constant of closed times ($\tau_S$; Fig. 3E, ○); 2) caused a fast mode of block with a time constant, $\tau_F = 0.83 \pm 0.08$ ($n = 6$), which did not vary with [flecainide] over the range 10–50 $\mu$M (not shown); and 3) decreased the mean open time ($\tau_o$; Fig. 3F, ○). The fast closures caused the conversion of channel long-lasting openings present under control conditions to bursts of openings. Flecainide decreased the duration of these bursts from 36 to 12 ms at 50 $\mu$M with an IC$_{50}$ of 15 $\mu$M.

The effect of flecainide on $\tau_o$ and $\tau_F$ is similar to that seen in systolic [Ca$^{2+}$], suggesting that the fast mode of block is the same in systole and diastole. However, the [flecainide] dependencies of $\tau_S$ associated with the slow mode were different in systolic and diastolic [Ca$^{2+}$] (Fig. 3E, cf. dashed line and ●), the latter showing a positive [flecainide] dependence. This
could be explained by Ca²⁺ affecting the reaction rates of the slow mechanism and/or an apparent lengthening of closed events due to the superposition of slow-mode closures and the long deactivated states of the channel that occur at subactivating [Ca²⁺]. Distinguishing these possibilities requires a direct measurement of the duration of individual slow-mode blocked events, which is not possible when they are masked by these deactivated RyR2 closures.

To more directly measure the duration of slow-mode block, we eliminated the masking by the RyR2-deactivated states by adding 5 mM caffeine to the cytoplasmic bath to increase the channel activation at 0.1 mM Ca²⁺ (Fig. 3B, top trace). Addition of 50 µM flecainide under these conditions induced short and long closures of the channel (bottom trace) with closed-time histograms shown in Fig. 3D. The time constant, $t_s$, was similar to that in the absence of caffeine (Fig. 3E) but different from that at high Ca²⁺ concentrations (Fig. 3E, dashed line), suggesting that differences in the reaction rates contribute to the different time constants of the slow mode of block at diastolic and systolic [Ca²⁺].

**Slow Mode of Flecainide Block Is Specific for RyR2 Closed States.** Figure 4 summarizes the on and off rates for flecainide block by fast and slow modes under different channel-activating conditions. The rates were derived from dwell-time histograms as described in the caption to Fig. 4. The on rate of the slow blocking mode was markedly reduced under conditions where RyR2 are strongly activated by cytoplasmic [Ca²⁺] compared with RyR2 at subactivating [Ca²⁺] (Fig. 4A, 0.1 mM Ca²⁺) or where RyR2 are subjected to Ca²⁺ or Mg²⁺ inhibition (Fig. 4A, 1 mM Ca²⁺ or Mg²⁺). This suggests that the slow mode of block has a preference for the inactive RyR2 channel. The rates associated with the fast mode (Fig. 4B) had a relatively weak dependence on the activating conditions compared with those for the slow mode. Nonetheless, the fast mode off rate was 2- to 3-fold lower at 100 µM to 1 mM cytoplasmic Ca²⁺ plus 1 mM Mg²⁺. The legend in (F) applies to (E).

*Significant difference from zero flecainide ($P < 0.05$).
of channel activity that occurred between the slow-mode blocked events. Flecainide on rate exhibited a strong positive correlation with $P_c$ under a range of activating conditions (Fig. 5B). Moreover, the correlations were similar for all activating conditions except for cytoplasmic solutions containing 1 mM Ca$^{2+}$ and Mg$^{2+}$. For the fast-mode, on rate, $R_{Fon} = 1/t_{Fon}$ and $R_{Foff} = 1/t_{Foff}$. For the slow-mode, on rate, $R_{Son} = 1/t_{Son}$ and $R_{Soff} = 1/t_{Soff}$. Note that slow-mode inhibition was not resolved in 0.1 mM Ca$^{2+}$ plus caffeine in (A) and 0.1 mM Ca$^{2+}$ plus caffeine in (B) ($\ast P < 0.05$; $** P < 0.01$).

The proposed scheme is also consistent with the [flecainide] dependencies of slow-rate modes in Fig. 5C, where the on rate for slow inhibition increased proportionally with flecainide concentration and the off rate showed no significant dependence on concentration. The scheme is also consistent with [Mg$^{2+}$] dependence of the rate constants for the slow mode ($k_{Son}$ and $k_{Soff}$; Fig. 5D), where it can be seen that $k_{Son}$ and $k_{Soff}$ did not show any dependence on [Mg$^{2+}$], thus confirming that these can be considered as constants describing the interaction of Mg$^{2+}$ and flecainide on RyR2.

**Effect of pH on Flecainide Inhibition.** Flecainide is a monovalent cation at neutral pH with a positive charge on its amide group ($pK_a = 9.2$) (Liu et al., 2003). At pH 7.4, 99% of the molecules are in the charged, protonated form, whereas at pH 9.5, this fraction is reduced to 40%. To know which form of flecainide is responsible for RyR2 inhibition, we compare the rates of fast and slow modes of flecainide inhibition at pH 7.4 and 9.5, as shown in Fig. 6. The recordings in Fig. 6, A and B show that raising cytoplasmic pH from 7.4 to 9.5 markedly decreased the inhibition caused by 50 μM flecainide. This is shown for both the fast mode in Fig. 6A (in the absence of Mg$^{2+}$) and for the slow mode in Fig. 6B, where 1 mM Mg$^{2+}$ is present. Dwell-time histograms (Fig. 6, C and D) demonstrate that raising cytoplasmic pH from 7.4 to 9.5 attenuates the frequency of both short and long exponential components of the flecainide-induced closures. This occurred via a 6-fold reduction in the on-rate constant of the slow mode (Fig. 6E) and a 2-fold reduction in the fast mode (Fig. 6F). The loss of potency at high pH indicates that the cationic form of flecainide mediates the bulk of slow and fast modes of block.

The relative effects of cytoplasmic and luminal pH on flecainide block were used to gain clues about the side (i.e., cis or trans) of action of flecainide on the RyR2. This is difficult to determine from flecainide alone because it appears to readily cross the bilayer. It is apparent from Fig. 6, E and F that the on rates of slow and fast block for flecainide applied from the cytoplasmic side were sensitive to cytoplasmic pH and not luminal pH. Moreover, increasing cytoplasmic pH could attenuate fast-mode block by flecainide applied from the luminal side (Fig. 6G). Together, these results indicate that flecainide only has access to its site of action from the cytoplasmic side.

**Substate Block by Flecainide Fast Mode.** Single-channel recording of the fast mode of block at +60 mV in the presence of 100 μM cytoplasmic Ca$^{2+}$ and 2 mM ATP revealed that it was due to flecainide-induced transitions from the RyR2 open state to a conductance substate and flecainide-induced transitions from the substate to the closed state (Fig. 7A). Substate current level did not depend on flecainide concentration. The current/voltage relationships (Fig. 7B) indicate a slope conductance of 87 ± 5 pS for the substate and 525 ± 10 pS for the open state, which was identical to that of the open state in the absence of flecainide.

We tested the hypothesis that the substate was due to the binding of a single flecainide molecule to the RyR2, and that the brief closures were due to the binding of two molecules as shown by the scheme in Fig. 7C. The scheme predicts that transition rates from open state to substate and from substate to closed state are proportional to flecainide concentration, whereas the corresponding reverse transition rates are concentration-independent. The concentration dependencies of rates shown in Fig. 7D show that indeed this is the case. The transition rates for the fast inhibition were calculated using the hidden Markov model algorithm (Materials and Methods).

**Voltage Dependence of Flecainide Inhibition.** The voltage dependence of the flecainide rate constants may provide clues to the location of its binding sites on the RyR2 molecule within the transmembrane electric field. The flecainide slow-mode on-rate constant was strongly voltage-dependent, whereas the off-rate constant had no voltage dependence (Fig. 8A). For the fast mode, the flecainide rate constants were all voltage-dependent (Fig. 8B). Transitions between open and substates
than the cytoplasmic bath. For the fast mode of flecainide slow mode that is much closer to the luminal bath potential a value of 20-fold and decreased the off-rate constant (relative positions of the flecainide binding sites (is related to the ionic charge of flecainide (model, the voltage dependence of the flecainide rate constants to the voltage dependence of the electrostatic potential 1973) attributes the voltage dependence of each binding con-

\[ k_{on} = k_{on}(0) \exp(\delta zFV/RT) \] and \[ k_{off} = k_{off}(0) \exp(\delta b - \delta zFV/RT) \]

where \( V \) is the bilayer potential, \( k_{on}(0) \) and \( k_{off}(0) \) are the binding constants at zero volts, and \( F, R, \) and \( T \) have their usual meanings. Values of \( \delta \) are derived from the slopes of the log-linear plots shown in Fig. 8. For cytoplasmic flecainide, \( \delta = 0 \) would indicate a location on the cytoplasmic side of the membrane and \( \delta = 1 \), the luminal side. For the slow inhibition, the voltage dependence of the ratio \( k_{on}/k_{off} \) gives a value of \( \delta = 1.15 \pm 0.14 \), suggesting a binding site for the slow mode that is much closer to the luminal bath potential than the cytoplasmic bath. For the fast mode of flecainide block, the voltage dependencies of the rate constants were not readily interpreted within the framework of the Woodhull model because the slopes of the voltage dependencies varied over the experimental voltage range (\( \delta \) ranged from 0 to 1.4 \pm 0.4) and so did not give a unique value for \( \delta \).

**[Cs\(^+\)]\(^-\)** Dependence of Flecainide Inhibition. We measured the effect of reducing [Cs\(^+\)] from 250 to 150 mM to match the physiologic ionic strength of the cytoplasm, and found that the IC\(_{50}\) for flecainide block was reduced ~5-fold (Fig. 1C). In Fig. 9, we examined the effect of this change in [Cs\(^+\)] on rate constants for fast and slow modes of flecainide block. The rate constants for fast inhibition were insensitive to [Cs\(^+\)], but reducing [Cs\(^+\)] from 250 to 150 mM caused a 2.5-fold decrease in the off-rate constant for the slow mode. However, the change in off-rate constant would account for only half (2.5-fold) of the decrease in the IC\(_{50}\) seen in Fig. 1C. An additional contribution to the effect of [Cs\(^+\)] on the IC\(_{50}\) may arise from the reduced channel \( P_{o} \), in 150 mM [Cs\(^+\)] (0.75 in 250 mM reducing to 0.37 in 150 mM; see \( P_{o} \) max in Table 1), leading to an increase in the on rate for the closed-state block as shown in Fig. 5, A and B. Therefore, the increased potency of flecainide at lower [Cs\(^+\)] is likely due to both a reduction in the off rate for the closed-state, slow block and an increase in the closed-state probability of the channel.

**Discussion**

This study presents a detailed analysis of the kinetics of RyR2 inhibition by flecainide, and its dependence on the activation state of the channel and the overall scheme derived for inhibition is shown in Fig. 10. We show that two modes of
The kinetics of flecainide block at diastolic cytoplasmic $[\text{Ca}^{2+}]$ are different from those seen under systolic $[\text{Ca}^{2+}]$ (cf. Figs. 2 and 3). The time constants for the dwell times associated with the fast mode of inhibition are similar in diastolic and systolic conditions, suggesting that the fast mode operates in a similar way under both systolic and diastolic conditions. However, the kinetics of the slow mode measured in systolic $[\text{Ca}^{2+}]$ do not account for the properties of the slow-mode inhibition at diastolic $[\text{Ca}^{2+}]$ in two respects: 1) the $[\text{flecainide}]$ dependencies of long closed times ($\tau_S$) are very different (Fig. 3E), and 2)
the on-rate constant for slow block in systolic \([\text{Ca}^{2+}]\) (\(k_{\text{Son}} = 0.05 \text{ Ms}^{-1}\); Fig. 8A at +40mV) is 20-fold too slow to account for the reduction in burst duration (36–12 ms) brought about by 50 \(\mu\text{M}\) flecainide at diastolic \([\text{Ca}^{2+}]\). The \([\text{flecainide}]\)-dependent reduction in burst duration reported here and previously (Hilliard et al., 2010) indicates another, faster mechanism of inhibition that might be an increased deactivation rate of the RyR2 channel caused by flecainide (i.e., the deactivation transition in Fig. 10).

The anesthetics procaine and the quaternary amine derivatives of lidocaine (QX314, QX222) have been shown to act on RyR2 only when applied to the cytoplasmic side of the membrane (Tinker and Williams, 1993; Xu et al., 1993). However, determination of the side of the membrane from which flecainide binds to the RyR2 is complicated by the fact that flecainide is membrane-permeable. For example, in cardiomyocytes, flecainide must enter the cell to reach its site of action on Nav1.5 channels (Strichartz, 1973; Wang et al., 1995). In our bilayer experiments, flecainide, when applied to either side of the membrane, exhibited the same voltage-dependent modes of block, suggesting that flecainide readily crosses the bilayer and that cytoplasmic and luminal flecainide act by a common mechanism. The 3-fold lower potency for flecainide on the luminal side (relative to cytoplasmic) and the fact that flecainide inhibition was only sensitive to cytoplasmic pH indicate that the flecainide site of action is only accessible from the cytoplasmic solution.

We observed distinctly different \([\text{Cs}^{+}]\) and voltage dependencies of the flecainide on and off rates associated with fast and slow block (Figs. 8 and 9), suggesting that the fast and slow modes operate via different binding sites. However, the mechanisms for the \([\text{Ca}^{2+}]\) and voltage dependencies are not yet clear. The Woodhull model (Woodhull, 1973) does not readily explain the nonexponential voltage dependence of the rate constant for the fast mode of block (Fig. 8B). An alternative model has been proposed to explain the voltage dependence of binding of uncharged drugs to RyR2 such as amitriptyline and ryanodols (Tanna et al., 2000; Chopra et al., 2009). In that model, the voltage dependence of energy barrier profiles is due to distortion of the binding site by the electric field. It is possible that both mechanisms contribute to the observed voltage dependence of flecainide block.

The fast RyR2 blocking mode of flecainide appears to be a common property of local anesthetics. Cocaine (Tsushima et al., 1996), quinidine, quinadinium (Tsushima et al., 2002), and the quaternary amine derivative of lidocaine (QX314; Xu et al., 1993) inhibit RyR2 with a flicker block comprising a conductance substate and complete channel closures. The voltage dependence of fast flecainide inhibition has an effective valency (\(\delta\)) of 1.4, which is similar to that reported for quinidine (1.2; Tsushima et al., 2002) and QX314 (1.4; Tinker et al., 1992). These voltage dependencies were seen as evidence that the local anesthetics bind in the channel pore. However, the strongest evidence for this comes from mutagenesis studies in Na\(^+\) and K\(^+\) that show amino acid residues near the cytoplasmic end of the pore channels are important for binding of local anesthetics, including flecainide (Ragsdale et al., 1994, 1996; Yeola et al., 1996). The slow block of RyR2 has also been reported for tetracaine (Xu et al., 1993; Laver and
In Nav1.5 channels, three local anesthetic blocking mechanisms have been identified (Sheets et al., 2010): 1) an open/inactivated-state block associated with a reduction in the gating charge and stabilization of the S4 segments, 2) a resting closed-state block that may involve a diffuse binding region in the inner vestibule of the pore, and 3) a fast-flicker block due to interaction of the charged forms of local anesthetics with the cytoplasmic side of the selectivity filter. The slow and fast modes for flecainide block of RyR2 have similar characteristics as the latter two forms of Na<sup>+</sup> channel block (items 2 and 3, respectively).

Under systolic conditions, tetracaine shares very similar RyR2 inhibition mechanisms with flecainide (Laver and van Helden, 2011). They both have fast and slow modes of block where only the slow mode has a preference for the closed RyR2 confirmation. This raises a question as to why drugs with such similar mechanisms of action have such different effects on Ca<sup>2+</sup>-release events. Flecainide increases Ca<sup>2+</sup> spark frequency but reduces their amplitude, whereas tetracaine only reduces spark frequency. Moreover, flecainide reduces the occurrence of Ca<sup>2+</sup> waves, whereas tetracaine increases Ca<sup>2+</sup> wave amplitude (Hilliard et al., 2010). The answer may lie in the relative off rates of flecainide and tetracaine associated with their slow modes of block. It has been proposed that tetracaine destabilizes SR Ca<sup>2+</sup> release because it is a more potent inhibitor of Ca<sup>2+</sup> release during diastole. This would increase diastolic Ca<sup>2+</sup> loading of the SR, leading to an increase in systolic Ca<sup>2+</sup> release when tetracaine block is alleviated (Laver and van Helden, 2011). During the course of the heartbeat, the relatively fast off rate of tetracaine (~20 seconds<sup>-1</sup>) will allow substantial loss in its blocking potency during systole. This would effectively increase the feedback on SR Ca<sup>2+</sup> release generated by cytoplasmic and luminal Ca<sup>2+</sup> and promote instability of Ca<sup>2+</sup> release, whereas the slower off rate for flecainide (~1 second<sup>-1</sup>) will tend to sustain flecainide block throughout systole.

The better antiarrhythmic efficacy of flecainide versus tetracaine may lie in its different blocking kinetics under diastolic conditions. Flecainide reduces burst duration of RyR2 activity at diastolic cytoplasmic [Ca<sup>2+</sup>], whereas tetracaine does not (Hilliard et al., 2010). However, it needs to be emphasized that, in addition to RyR2 inhibition, Na<sup>+</sup> channel block by flecainide is clearly important for its efficacy in CPVT. Reducing the Na<sup>+</sup> current can 1) inhibit action potentials triggered by sodium-calcium exchanger–mediated depolarizations (Watanabe et al., 2009), and 2) increase sodium-calcium exchanger–mediated Ca<sup>2+</sup> efflux, leading to a decrease in Ca<sup>2+</sup> concentration in the vicinity of RyR2 (Sikkel et al., 2013; Steele et al., 2013).

In conclusion, we find that in systolic [Ca<sup>2+</sup>], flecainide causes RyR2 closures via independent fast and slow modes.
The slow mode has a preference for the closed RyR2 confirmation, whereas the fast mode does not depend on RyR2 state. Flecainide accesses its sites of action for both modes of inhibition from the cytoplasmic side of the membrane. In diastolic [Ca\textsuperscript{2+}], there is another flecainide inhibition mechanism causing an open-channel block, in addition to the fast and slow modes.

Acknowledgments

The authors thank Paul Johnson for his assistance with the single-channel recording.

Authorship Contributions

Participated in research design: Mehra, Laver.

Conducted experiments: Mehra.

Contributed new reagents or analytic tools: Laver.

Performed data analysis: Mehra, Laver.

Wrote or contributed to the writing of the manuscript: Knollmann, Mehra, Imtiaz, van Helden, Laver.

References


Campbell TJ and Vaughan Williams EM (1983) Voltage- and time-dependent deactivation of cardiac Na\textsuperscript{+} current and diastolic [Ca\textsuperscript{2+}] in the sheep cardiac sarcoplasmic reticulum. *J Physiol* 339:531–540.


Tinker A, Lindsay AR, and Williams AJ (1992) Large tetraethyl ammonium cations produce a reduced conductance state in the sheep cardiac sarcoplasmic reticulum Ca\textsuperscript{2+} release channel. *Biophys J* 61:1112–1122.


Address correspondence to: Dr. Derek Laver, School of Biomedical Sciences and Pharmacy, University of Newcastle and Hunter Medical Research Institute, Callaghan, NSW 2308, Australia. E-mail: Derek.Laver@newcastle.edu.au
Multiple modes of RyR2 inhibition by flecainide

D. Mehra, M.S. Imtiaz, D.F. van Helden, B.C. Knollmann and D.R. Laver

Molecular Pharmacology

Supplemental Figure. Dwell-time analysis of luminal flecainide inhibition. (A) Closed dwell-time histograms showing the rate of closed events based on the method of Sigworth and Sine (1987) as described in the text. (B) Corresponding open dwell-time histograms. The legend applies to A and B.