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Flecainide inhibits arrhythmogenic Ca^{2+} waves by open state block of ryanodine receptor Ca^{2+} release channels and reduction of Ca^{2+} spark mass

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Abstract

Catecholaminergic polymorphic ventricular tachycardia (CPVT) is linked to mutations in the cardiac ryanodine receptor (RyR2) or calsequestrin. We recently found that the drug flecainide inhibits RyR2 channels and prevents CPVT in mice and humans. Here we compared the effects of flecainide and tetracaine, a known RyR2 inhibitor ineffective in CPVT myocytes, on arrhythmogenic Ca^{2+} waves and elementary sarcoplasmic reticulum (SR) Ca^{2+} release events, Ca^{2+} sparks. In ventricular myocytes isolated from a CPVT mouse model, flecainide significantly reduced spark amplitude and spark width, resulting in a 40% reduction in spark mass. Surprisingly, flecainide significantly increased spark frequency. As a result, flecainide had no significant effect on spark-mediated SR Ca^{2+} leak or SR Ca^{2+} content. In contrast, tetracaine decreased spark frequency and spark-mediated SR Ca^{2+} leak, resulting in a significantly increased SR Ca^{2+} content. Measurements in permeabilized rat ventricular myocytes confirmed the different effects of flecainide and tetracaine on spark frequency and Ca^{2+} waves. In lipid bilayers, flecainide inhibited RyR2 channels by open state block, whereas tetracaine primarily prolonged RyR2 closed times. The differential effects of flecainide and tetracaine on sparks and RyR2 gating can explain why flecainide, unlike tetracaine, does not change the balance of SR Ca^{2+} fluxes. We suggest that the smaller spark mass contributes to flecainide's antiarrhythmic action by reducing the probability of saltatory wave propagation between adjacent Ca^{2+} release units. Our results indicate that inhibition of the RyR2 open state provides a new therapeutic strategy to prevent diastolic Ca^{2+} waves resulting in triggered arrhythmias, such as CPVT.

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Keywords

Calcium sparks; flecainide; tetracaine; RyR2; catecholaminergic polymorphic ventricular tachycardia

Introduction

In cardiac excitation-contraction coupling, transmembrane Ca^{2+} flux via L-type Ca^{2+} channels triggers Ca^{2+} release from RyR2 Ca^{2+} release channels located in the junctional SR.[1] The SR Ca^{2+} content is determined by the net balance of efflux via RyR2 channels and Ca^{2+} uptake via the SR Ca^{2+} ATPase (SERCA), also referred to as leak-pump balance.[2] Agents that increase RyR2 channel open probability and Ca^{2+} efflux (e.g. caffeine) cause a decrease in SR Ca^{2+} content, whereas RyR2 inhibitors (e.g. tetracaine) increase SR Ca^{2+} content in intact myocytes during steady-state pacing.[3,4] However, despite these changes in SR Ca^{2+} content, agents that alter the open probability of RyR2 typically have no effect on the steady-state Ca^{2+} transient due to compensatory changes in fractional Ca^{2+} release.[5]

Mutations in RyR2 or calsequestrin (Casq2), the major SR Ca^{2+} binding protein, can cause an inherited arrhythmia syndrome, catecholaminergic polymorphic ventricular tachycardia (CPVT).[6,7] Ventricular myocytes isolated from mouse models of CPVT exhibit catecholamine-induced premature SR Ca^{2+} release and spontaneous Ca^{2+} waves that trigger delayed afterdepolarizations and premature beats.[8,9] These Ca^{2+} waves are likely responsible for triggering ventricular arrhythmias *in vivo*.[10] We recently found that the anti-arrhythmic drug flecainide blocks RyR2 channels, reduces Ca^{2+} wave frequency in Casq2-/- cardiomyocytes and prevents CPVT in mice and humans.[11] In contrast, the RyR2 channel inhibitor, tetracaine, was ineffective and did not suppress Ca^{2+} waves in Casq2-/- myocytes during prolonged exposure.[11] Surprisingly, unlike tetracaine, flecainide did not cause the increase in SR Ca^{2+} content expected for RyR2 channel inhibitors, in apparent violation of the principle of SR Ca^{2+} flux balance.[12,13]

Here we test the hypothesis that flecainide prevents arrhythmogenic Ca^{2+} waves by altering the properties of elementary SR Ca^{2+} release events, Ca^{2+} sparks.[14] We found that in contrast to tetracaine, flecainide reduced spark mass but increased spark frequency. As a result, flecainide had no net effect on spark mediated Ca^{2+} leak, which would explain why flecainide does not affect SR Ca^{2+} leak pump balance or the SR Ca^{2+} content. The different effects of flecainide and tetracaine on Ca^{2+} sparks are likely the result of differential block of RyR2 channels: flecainide only inhibits open RyR2 channels and reduced RyR2 channel open duration, whereas tetracaine blocks RyR2 channels by reducing the rate of RyR2 channel openings, but has no effect on open RyR2 channels. Thus, our data suggest that the mode of RyR2 channel inhibition (e.g. open channel versus closed channel block) determines the anti-arrhythmic efficacy of RyR2 inhibitors.

Methods

The use of animals in this study was approved by the Animal Care and Use Committees of Vanderbilt University in the USA, University of Leeds in the UK, and University of Newcastle in Australia.

Experiments on intact myocytes from Casq2-/- mice

Ventricular myocytes from 3-4 month old *Casq2-/- mice* were isolated by a modified collagenase/protease method as previously described.[8] All chemicals, unless otherwise specified were obtained from Sigma (St. Louis, MO). All the experiments were conducted in

Tyrode's solution containing (in mM): NaCl 134, KCl 5.4, $MgCl_2$ 1, glucose 10, and HEPES 10, pH adjusted to 7.4 with NaOH. For experiments using field-stimulation, ventricular myocytes were loaded with Fura-2AM, and cytosolic [Ca^{2+}] estimated from the ratio of fluorescence emitted at 340 and 380 nm excitation (Fratio) as described.[8] For Ca^{2+} spark measurement, myocytes were incubated in Tyrode's solution containing 1 mM Ca^{2+}, 6.6 μM fluo-4 acetoxymethyl ester (fluo-4 AM) and 0.16 % Pluronic F127 for 20 minutes at room temperature to load the indicator in the cytosol. The supernatant was removed and myocytes were washed once with 1 mM Ca^{2+} Tyrode's solution. A minimum of 30 minutes were allowed for de-esterification of the indicator before imaging the cells. Aliquots of fluo-4 loaded myocytes were then incubated for 10 min in petri-dishes containing one of three experimental solutions: vehicle (0.1 vol% ethanol), flecainide (6 μM) or tetracaine (50 μM). Flecainide and tetracaine were dissolved in ethanol stock solutions to achieve a final ethanol concentration of 0.1 vol%. All experimental solutions contained 2 mM Ca^{2+} Tyrode's solution and 100 nM isoproterenol (ISO). In all experiments on mouse cells, Ca^{2+} sparks were detected in line scan mode using a Zeiss LSM 510 microscope, with the scan line positioned along the longitudinal axis of each cell. A subset of cells was rapidly exposed to 10 mM caffeine to assess the SR Ca^{2+} content under each condition. Cells were illuminated at 488 nm and emitted fluorescence was measured at >515 nm. Scan lines were 512 μm in length and collected at 1.60 ms intervals.

Experiments on permeabilized rat myocytes

Ventricular myocytes isolated from adult Wistar rats (150-200 g) were permeabilized by exposure to saponin (10 μg/ml) in a mock intracellular solution for 6 minutes, before centrifugation and re-suspension. Unless otherwise stated, chemicals used following permeabilization were obtained from the Sigma Chemical Corporation, Dorset, UK. Permeabilized cells were perfused with weakly Ca^{2+} -buffered solutions approximating to the intracellular milieu and SR Ca^{2+} release was detected using fluo-3. The basic solution contained (mM): KCl, 100; HEPES, 25; EGTA, 0.05-0.36; phosphocreatine 10; ATP, 5 and fluo-3 (pentapotassium) salt, 0.002, pH 7.1, 22 °C. $MgCl_2$ was added (from 1 M stock solution) to produce a free [Mg^{2+}] concentration of 1.0 mM. The free [Ca^{2+}] was adjusted by addition of $CaCl_2$.

The apparatus used for [Ca^{2+}] measurement in permeabilized cells has been described previously.[15] Briefly, permeabilized cells were placed in a cylindrical bath (5 mm diameter) in a Perspex block. The bottom of the bath was formed by attaching a coverslip to the underside of the block. A drop of solution containing cells was placed in the bath and a tightly fitting Perspex column inserted into the well until the lower surface was close to myocytes resting on the coverslip. Perfusion was achieved by pumping solution (0.3 ml/min) down a narrow bore running longitudinally through the column. After obtaining line scans in vehicle-containing solutions, myocytes were exposed for 2 min to flecainide (25 μM) or tetracaine (50 μM) and the line scans repeated. In permeabilized cells, the drug can enter rapidly and therefore, the exposure times were shorter than in the experiments with intact myocytes. Flecainide accumulates in the heart[11], which explains why higher drug concentrations were required to achieve a significant effect permeabilized myoyctes compared to intact myocytes.

The chamber was placed on the stage of a Nikon Diaphot Eclipse TE2000 inverted microscope and cells were viewed and illuminated using a confocal laser-scanning unit (Microradiance, Bio-Rad, Herts, UK) via a 60X water immersion lens (Plan Apo,NA 1.2). The dye was excited at 488 nm and emitted fluorescence was measured at >515 nm.

Analysis of confocal images

In both intact and permeabilized cell experiments, confocal data were analyzed using ImageJ (NIH, USA, http://rsbweb.nih.gov/ij/). The automated detection of Ca^{2+} sparks and the

measurement of temporal and spatial spark properties was carried out using the "SparkMaster" plugin for ImageJ with human verification of spark identification. The detection criteria were set to 3.8; *i.e.*, the threshold for the detection of events was 3.8 times the standard deviation of the background noise divided by the mean.[16]

Single RyR2 channel measurements

SR vesicles containing RyR2 channels were obtained from sheep hearts and were reconstituted into artificial lipid bilayers as previously described.[17] Lipid bilayers were formed from phosphatidylethanolamine and phosphatidylcholine (8:2 wt/wt, Avanti Polar Lipids, Alabaster, AL) in n-decane, (50 mg/ml, ICN Biomedicals) across an aperture of 150–250 μm diameter in a Delrin cup which separated two baths (*cis* and *trans*). During SR-vesicle incorporation, the *cis* (cytoplasmic) bath contained (in mM) 250 Cs^+ (230 $CsCH_3O_3S$, 20 CsCl), 1.0 $CaCl_2$ and 500 mannitol, while the *trans* (luminal) solution contained 50 Cs^+ (30 $CsCH_3O_3S$, 20 $CsCl_2$) and 1.0 $CaCl_2$. After detection of channels in the bilayer the compositions of the *cis* and *trans* solutions were changed as follows. The [$CsCH_3O_3S$] in the *trans* solution was increased to 230 mM (*i.e.* establishing 250 mM Cs^+ in both *cis* and *trans* baths) by means of aliquot addition of 4 M stock. The cytoplasmic solution was changed to one containing 2 mM ATP, 0.1 μM free Ca^{2+} by adding aliquots of ATP and BAPTA (4.5 mM) to the *cis* bath. The caesium salts were obtained from Aldrich Chemical Company, mannitol was obtained from Ajax chemicals and $CaCl_2$ from BDH Chemicals. Solutions were pH-buffered with 10 mM N-tris [Hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES, ICN Biomedicals) and solutions were titrated to pH 7.4 using CsOH (optical grade, ICN Biomedicals) and were redox buffered with 5 mM glutathione (ICN Biomedicals). Flecainide (acetate salt) and tetracaine (hydrochloride) were obtained from Sigma.

Bilayer potential was controlled using an Axopatch 200B amplifier (Axon Instruments). Electrical potentials are expressed using standard physiological convention (*i.e.*, cytoplasmic side relative to the luminal side at virtual ground). Single channel recordings were obtained using bilayer potential difference of +40 mV. The current signal was digitized at 10 kHz and low-pass filtered at 2 kHz with a Gaussian digital filter. Open probability (P_o) as well as open and closed durations was measured by the 50% threshold detection method. The burst properties of RyR2 openings (i.e. mean burst duration, intraburst Po and interburst gap durations) were derived from exponential fits to dwell-time distributions of channel open and closured intervals. Analysis was carried out using Channel2 software (P.W. Gage and M. Smith, Australian National University, Canberra).

Statistical analysis

All experiments were done in random sequence with respect to the drug, and measurements were taken by a single observer who was blinded to the drug treatment. Differences between groups were assessed using a one-way analysis of variance (ANOVA). If statistically significant differences were found, individual groups were compared with *Student's t*-test. Results were considered statistically significant if the p-value was less than 0.05. Unless otherwise indicated, results are expressed as arithmetic means ± standard error of mean.

Results

Flecainide prevents premature Ca^{2+} waves without altering SR Ca^{2+} content

Upon stimulation with the β-adrenergic receptor agonist ISO, field-stimulated Casq2-/- myocytes exhibit spontaneous Ca^{2+} release resulting in Ca^{2+} waves that occur prior to the next pacing stimulus (Fig. 1A, arrows, top panel). Such premature Ca^{2+} waves have been the characteristic finding in cardiomyocytes isolated from murine CPVT models[8,9,18] and are thought to be the mechanism responsible for triggering premature beats and ventricular

tachycardia in vivo.[19] We previously reported that flecainide reduces the frequency of Ca^{2+} waves, but this was tested only in quiescent Casq2-/- cardiomyocytes.[11] We find that flecainide was even more effective in preventing premature Ca^{2+} waves in field-stimulated myocytes (Fig. 1A, lower panel). Compared to 90% of vehicle-treated myocytes, only 38% of flecainide-treated myocytes exhibited Ca^{2+} waves during the ~60 s pacing train. In myocytes where Ca^{2+} waves were still present, flecainide significantly reduced wave frequency by more than 50% compared to vehicle-treated myocytes (Fig. 1B). At the same time, flecainide had no significant effect on diastolic [Ca^{2+}] (vehicle: 1.98±0.06 F_{ratio}, n=27, flecainide 1.91±0.07 F_{ratio}, n=26, p=0.5) or on SR Ca^{2+} content (Fig. 1C), which was estimated by rapid caffeine application after the pacing train (Fig. 1A). We next tried tetracaine, a RyR2 channel blocker which does not prevent Ca^{2+} waves in quiescent Casq2-/- myocytes during prolonged exposure.[11] Unfortunately, 50 μM tetracaine rendered myocytes unresponsive to field stimulation, making a direct comparison with flecainide impossible.

Flecainide reduces Ca^{2+} spark mass but increases Ca^{2+} spark frequency

We next compared the effect of flecainide and tetracaine on Ca^{2+} sparks (Fig. 2A) and the SR Ca^{2+} content, measured by rapid caffeine application (Fig. 2B). Compared to vehicle or tetracaine, flecainide significantly reduced the amplitude, duration and spatial width of Ca^{2+} sparks in *Casq2-/-* myocytes (Table 1 and Fig. 2A). On the other hand, tetracaine had no significant effect on spark amplitude or duration (Fig. 2A and Table 1). Interestingly, flecainide and tetracaine had the opposite effect on the frequency of spark occurrence: Flecainide significantly increased spark frequency, whereas tetracaine decreased it (Fig. 2C). Spark mass (calculated as amplitude × 1.206 × $FWHM^3$)[20] was significantly reduced by flecainide but not by tetracaine (Fig. 2D). As a result, the estimated spark-mediated leak was unchanged by flecainide, but significantly reduced by tetracaine (Fig. 2E). At the same time, flecainide had no significant effect on SR Ca^{2+} uptake rates (estimated from the decay time constant (τ) of field-stimulated Ca^{2+} transients (Vehicle 0.32 ± 0.08 s, n=19, vs flecainide 0.35 ± 0.10 s, n=20, p = n.s.). Ca^{2+} transient decline in mice predominantly depends on SERCA function.[21] Consistent with their differential effect on Ca^{2+} spark mediated leak, tetracaine increased SR Ca^{2+} content, whereas flecainide did not (Fig. 2F).

Flecainide prevents Ca^{2+} waves in permeabilized rat myocytes

The data from intact Casq2-/- myocytes suggest that flecainide reduced the probability of propagated waves by reducing spark Ca^{2+} mass. To test this hypothesis more directly, and at the same time exclude the possibility that the flecainide effect is limited to mouse Casq2-/- myocytes stimulated with ISO, we next compared flecainide and tetracaine in permeabilized rat ventricular myocytes, where there are no contributions from ion transport across the sarcolemma, cytosolic [Ca^{2+}] can be controlled experimentally, and ISO is not needed to induce Ca^{2+} waves. In the presence of 200 nM Ca^{2+} and 0.05 mM EGTA, permeabilized myocytes exhibited regular spontaneous Ca^{2+} waves (Fig. 3A), comparable with those observed in intact cells in the presence of ISO (Fig. 2A). Under these conditions, introduction of 25 μM flecainide (*upper*) or 50 μM tetracaine (*lower*) reduced the frequency of spontaneous Ca^{2+} waves by 42.8 ± 6.2 % (n=9) and 18 ± 4.6 % (n=6), respectively. However, in the presence of tetracaine, the amplitude of the spontaneous Ca^{2+} waves increased by 17.9 ± 7.1 % (n=7), while in the presence of flecainide, the amplitude decreased by 21.4 ± 2.1 (n=9). We next assessed the effects of flecainide and tetracaine on the SR Ca^{2+} content at the point of spontaneous SR Ca^{2+} release: In cells exhibiting spontaneous Ca^{2+} release at regular intervals, the frequency was monitored over 3-4 Ca^{2+} release and re-uptake cycles. Caffeine (20 mM) was then rapidly applied to maximally deplete SR Ca^{2+} at the point in the cycle when a spontaneous Ca^{2+} release would otherwise have occurred. The amplitude of the caffeine-induced Ca^{2+} transient was used as an assay of the SR Ca^{2+} content under control conditions, or in the presence of flecainide

or tetracaine (Fig. 3B). Tetracaine significantly increased the Ca^{2+} content of the SR at the point of spontaneous Ca^{2+} release, whereas flecainide had no significant effect.

In rat myocytes exhibiting repeated spontaneous Ca^{2+} waves, flecainide increased the frequency of Ca^{2+} sparks immediately before each release, although the events appeared of smaller amplitude and width (Fig 4A). In order to investigate the effects of flecainide on spontaneous Ca^{2+} sparks under more controlled conditions, the [EGTA] of the bathing solution was increased to a level that prevents propagation of Ca^{2+} waves (0.36 mM), without affecting spontaneous Ca^{2+} sparks.[22] Under these conditions, flecainide significantly increased spark frequency but decreased the spark mass by 45%, such that that there was no significant change in the spark mediated Ca^{2+} leak (Fig. 4B). The decrease in spark mass caused by flecainide was predominantly due to a decrease in spark width (Fig. 4B). In contrast to the effects of flecainide, tetracaine significantly decreased spark frequency, while spark width was unaffected. The mean spark amplitude also decreased slightly, resulting in a modest overall decrease in spark mass of ~12%, while the Ca^{2+} leak decreased by ~37% (Fig. 4).

Flecainide inhibits open RyR2 channels

To test the hypothesis that differential modulation of RyR2 channel gating is responsible for their different effects on Ca^{2+} sparks, we next compared the effect of flecainide and tetracaine on RyR2 channels in lipid bilayers. Under control conditions (1 mM luminal Ca^{2+}, 0.1 μM cytoplasmic Ca^{2+}), RyR2 had an open probability (Po) of 0.15 ± 0.04, mean open time (τ_o) of 46 ± 11 ms and mean closed time (τ_c) of 441 ± 111 ms, (n=14). The addition of flecainide to the cytoplasmic bath induced brief channel closures with a mean duration of ~1 ms to a substate of ~20% of the maximal channel conductance (Fig. 5A). These closures caused the conversion of channel long-lasting openings present under control conditions to bursts of openings (Fig. 5A). Although flecainide and tetracaine both inhibited RyR2, they did so with different mechanisms of action. Unlike flecainide, tetracaine did not induce short closures of the channel (Fig 5B). Moreover, tetracaine did not shorten the burst duration, as illustrated by the example in Fig 5B (right panel), where the mean burst duration was essentially equal to the mean open time.

Flecainide inhibited the Po with an IC_{50} of 16 ± 3 μM (Hill coefficient of 1.3 ± 0.8) whereas tetracaine inhibited Po with an IC_{50} of 44 ± 4 μM (Hill coefficient of 1.4 ± 0.5, Supplemental Fig. 1A). We next quantified the concentration dependence of the effect of flecainide and tetracaine on the closed time between bursts (Supplemental Fig. 1B), length of bursts (Supplemental Fig. 1C), and the open probability within bursts (Supplemental Fig. 1D). Flecainide was a more potent inhibitor of burst duration and burst Po compared with tetracaine. Both drugs had about equal potency in increasing interburst gap duration. To determine the mechanism responsible for the inhibition of RyR2, we next compared these effects of flecainide and tetracaine at drug concentrations that produced an approximately equal reduction in RyR2 Po (Fig 5C-F). For flecainide, the decrease in RyR2 Po was due to a 47% reduction in burst duration (Fig. 5D) and a 6% reduction in burst Po (Fig. 5E), whereas interburst closed duration (Fig. 5F) was not significantly different from control. Thus, the main inhibitory effect of flecainide was a shortening of the mean burst duration, as illustrated in Fig. 5A, right panel. For tetracaine, the decreased Po was mainly the result of increasing the mean closed duration (a 104% increase in the duration of interburst closures, Fig. 5F). Tetracaine had no significant effects on burst duration (Fig. 5D) and burst Po (Fig. 5E). Thus, flecainide inhibited RyR2 primarily by reducing mean burst duration, whereas tetracaine inhibited RyR2 by increasing the mean time channels remained closed between bursts. The different effects of tetracaine and flecainide on RyR2 channels are best illustrated by comparing burst mass (Fig. 6). Burst mass is the product of burst duration and open probability within bursts, analogous to calculating

spark mass in myocytes. At concentrations below ~2 × IC_{50}, tetracaine has no significant effect on burst mass whereas flecainide significantly reduced burst mass (Fig. 6).

Discussion

In this study we have compared the actions of two RyR2 blockers that had very different effects on Casq2-/- myocytes, a model of CPVT: Flecainide induced a sustained decrease in the frequency of spontaneous Ca^{2+} waves, whereas tetracaine was ineffective.[11] Here we demonstrate that tetracaine and flecainide have different inhibitory actions on single RyR2 channels, which result in markedly different effects on Ca^{2+} sparks, Ca^{2+} waves and the balance of SR and sarcolemmal Ca^{2+} fluxes. The mechanism of flecainide action is an open channel block. Flecainide consistently reduces spark mass in different species (mouse, rat). Flecainide exerts analogous effects in intact and permeabilized myocytes regardless of whether cells were treated with ISO or not, suggesting that the state of RyR2 phosphorylation is not a major determinant of flecainide action.

Regulation of RyR2 and the balance of SR and sarcolemmal Ca^{2+} fluxes

In paced ventricular myocytes, increasing the open probability of RyR2 with a low concentration of caffeine (~0.2 mM) initially increases the amplitude of the whole cell Ca^{2+} transient.[3,23] However, the increased Ca^{2+} transient amplitude results in a greater efflux of Ca^{2+} from the cell, which progressively depletes SR Ca^{2+} until Ca^{2+} transient return to control levels.[3,23] In the new steady state the SR Ca^{2+} content is decreased and fractional SR Ca^{2+} release is increased, reflecting the continuing effect of caffeine on RyR2. A decrease in the open probability of RyR2 induced by application of tetracaine produces essentially the opposite effect. Decreased Ca^{2+} transient amplitudes and reduced Ca^{2+} extrusion from the cell cause a progressive increase in the SR Ca^{2+} content until the Ca^{2+} transient amplitude is restored.[3] This tendency for SR Ca^{2+} release to “autoregulate” with changes in SR content is also apparent with spontaneous Ca^{2+} sparks[24] and reflects the effect of SR luminal Ca^{2+} on RyR2 open probability.[25]

Flecainide apparently violates these principles of SR autoregulation: In contrast to tetracaine, [26] RyR2 inhibition with flecainide suppressed spontaneous SR Ca^{2+} waves without causing a compensatory increase in SR load in paced ventricular myocytes (Fig. 1). At the same time, flecainide increased Ca^{2+} spark frequency in intact (Fig. 2) and permeabilized myocytes (Fig. 4), a result usually seen with RyR2 channel *activators* such as caffeine.[24] However, despite increasing spark frequency, flecainide significantly reduced Ca^{2+} wave frequency (Fig. 1+3), which is the opposite effect of caffeine.[27]

Unique regulation of RyR2 channels by flecainide

The paradoxical effects of flecainide on myocyte Ca^{2+} handling are likely the result of flecainide's unique action on RyR2 channels. Flecainide does not alter the RyR2 closed times (Fig. 5), which is in stark contrast to the effects of caffeine or tetracaine, the two compounds used most often by investigators studying the effect of RyR2 channel modulation on myocyte Ca^{2+} handling.[13,24,26-30] Both caffeine and tetracaine modulate RyR2 open probability by acting on closed RyR2 channels,[28,31] which likely explains their effect on spark frequency. [24] Although tetracaine can also decrease RyR2 open times in the presence of high cytoplasmic [Ca^{2+}],[11] at more physiological cytoplasmic [Ca^{2+}] examined here (0.1 μM Ca^{2+}) and previously by Gyorke and co-workers (3 μM Ca^{2+}),[28] tetracaine acts primarily by stabilizing closed channels (Fig. 5). Caffeine also does not alter RyR2 open times.[31]

In contrast, flecainide acts mainly as an open channel blocker. Flecainide decreases RyR2 open times and reduces RyR2 conductance by causing brief closures to a subconductance state (Fig.

5). As a result, flecainide is much more potent than tetracaine in reducing burst mass (Fig. 6). The reduction in RyR2 burst mass can explain flecainide's effect of decreasing spark mass in intact and permeabilized myocytes (Figs. 2+4). On the other hand, it is much less clear why flecainide increased spontaneous Ca^{2+} spark frequency (Fig. 2). We previously found that flecainide reduced both open and closed times of RyR2 channels activated by high cytosolic (100 μM) Ca^{2+},[11] which could explain the dual effect of reduced spark mass and increased spark frequency (Fig. 2). However, flecainide had no effect on closed times of RyR2 channels under experimental conditions that mimic spontaneous sparks in myocytes studied here (Fig. 5). Alternatively, the increased spark frequency may be the consequence of flecainide's effect on spark mass. Because spark mass is reduced, each spark results in a smaller reduction in SR luminal [Ca^{2+}]. The effect of luminal Ca^{2+} on RyR2 gating[25] is then analogous to a pressure overflow valve, triggered at a set pressure: In the presence of flecainide, smaller spontaneous sparks must now occur more frequently to match the luminal Ca^{2+} control signal, dictated by constant Ca^{2+} influx via SR Ca^{2+} ATPase.

Regardless of the underlying mechanism, flecainide had no effect on SR Ca^{2+} leak due to its opposing influence on spark frequency and spark mass, and SR Ca^{2+} content remained unchanged. The characteristic changes in Ca^{2+} spark properties induced by flecainide may also underlie its potency as an inhibitor of diastolic Ca^{2+} waves: The pronounced decrease in spark mass induced by flecainide likely reduces the probability of saltatory propagation between neighboring couplons. In that regard it is interesting to note that sparks measured in Casq2-/- myocytes (table 1) were about 50% wider and longer than those reported in the literature.[16] While the bigger spark mass can be in part explained by the presence of ISO,[32] we cannot exclude that loss of Casq2 also contributed to the large spark mass found in Casq2-/- myocytes.

Implications for therapeutic actions of RyR2 inhibitors

Our data suggests that the beneficial actions of flecainide stem from its peculiar ability to block RyR2 channels only while they are open, to cause blocking events with a sufficient frequency to reduce mean open times to ~1 ms, and sufficient brevity to avoid an increase in the interburst intervals. While many compounds are known to inhibit RyR2 activity,[33] relatively few of them have been studied at the single channel level. Among those, even fewer act as open channel blockers. For example, ryanoids (ryanodine analogues) are highly specific open channel blockers, but RyR2 inhibition is much slower than flecainide and occurs on the second timescale.[34] Polyamines such as neomycin are open channel blockers that operate by a "blocker-stopper" mechanism with fast kinetics.[35] Unfortunately, in the presence of diastolic Ca^{2+} levels, neomycin also acts as a closed channel blocker by competing with Ca^{2+} for a channel activation site.[36] Finally, quaternary ammonium ions have been shown to plug the RyR2 pore and act as fast open channel blockers.[37] However, the block has a relatively low affinity (~1 mM) and these compounds are well known K^+ channel blockers. Thus, the properties of flecainide represent a unique mechanism of modulating RyR2 channels, which is ideally suited to suppress arrhythmogenic Ca^{2+} waves without causing compensatory increases in SR Ca^{2+} content.

In summary, our results suggest that sustained inhibition of catecholamine-induced diastolic Ca^{2+} waves in CPVT myocytes requires a drug that interacts primarily with RyR2 in their open state to reduce spark mass, but without affecting the overall balance of SR Ca^{2+} fluxes. In the absence of changes in net Ca^{2+} fluxes or the SR Ca^{2+} content, slow compensatory changes in RyR2 function via autoregulation do not occur. This results in a sustained therapeutic action which is reflected by a reduced probability of saltatory wave propagation due to a smaller spark mass. Our data suggests a new paradigm for anti-arrhythmic drug development targeting RyR2 channels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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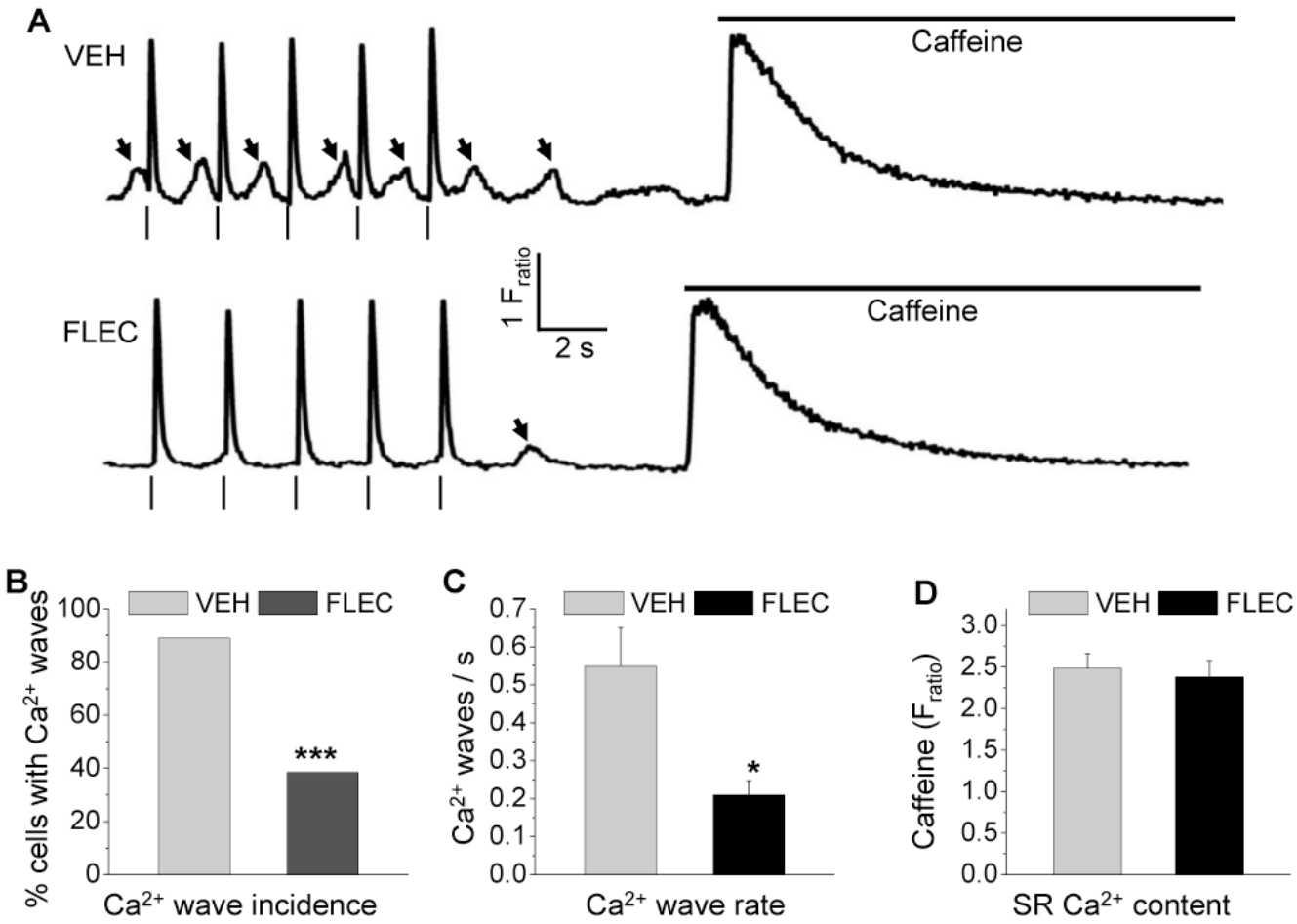

Fig. 1.

Flecainide prevents premature Ca^{2+} waves.

(A) Representative examples of cytosolic Ca^{2+} fluorescence recordings from field-stimulated *Casq2-/-* myocytes loaded with the fluorescent indicator fura2AM. Arrhythmogenic Ca^{2+} waves (arrows) were induced by 1 μM ISO in the bath solution. SR Ca^{2+} content was quantified by rapid caffeine (10 mM) 0.5 Hz pacing train. In vehicle treated cells (VEH), spontaneous Ca^{2+} waves followed each application following the paced beat (|). Pretreatment with flecainide (FLEC, 6 μM) prevented the generation of Ca^{2+} waves during the pacing train.

(B) Fraction of *Casq2-/-* myocytes that exhibit ISO-induced Ca^{2+} waves during the pacing train. VEH n=27, FLEC n=26, ***p<0.001 by Fisher-exact test.

(C) Comparison of average rate of Ca^{2+} waves during pacing train. Only myocytes that exhibited Ca^{2+} waves were included in the analysis. VEH n=24, FLEC n=10, *p<0.05, ***p<0.001.

(D) Comparison of average SR Ca^{2+} content after the pacing train. VEH n=27, FLEC n=26 myocytes.

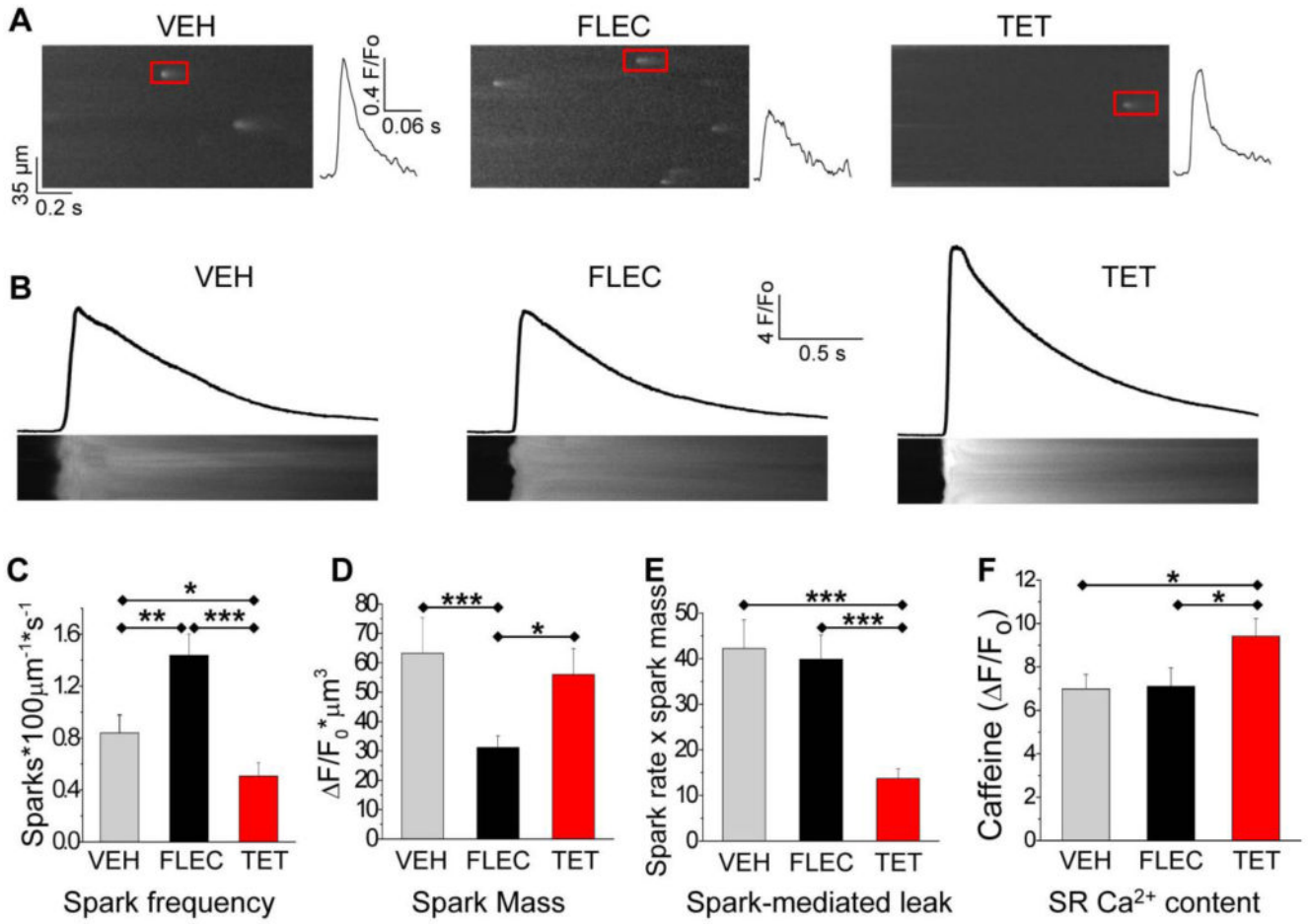

Fig. 2.

Differential effect of flecainide and tetracaine on Ca^{2+} sparks and SR Ca^{2+} content in intact Casq2-/- myocytes.

(A) Representative line scans and individual Ca^{2+} sparks (red box) of quiescent Casq2-/- myocytes loaded with Fluo-4AM. To avoid excessive Ca^{2+} wave generation, ISO concentration was reduced to 0.1 µM in the bath solution. Myocytes were incubated for 10 min with either vehicle (VEH), flecainide (FLEC, 6 µM) or tetracaine (TET, 50 µM) before obtaining the confocal images. Red box: spark plot to the right

(B) Representative line scans during rapid caffeine application. The amplitude of the caffeine transient was used as an estimate of SR Ca^{2+} load.

(C-F) Comparison of average spark frequency (C), spark mass (D), spark mediated SR Ca^{2+} leak (=spark mass × spark frequency) and SR Ca^{2+} content (F). n=77-97 myocytes per group, *p<0.05, ***p<0.001

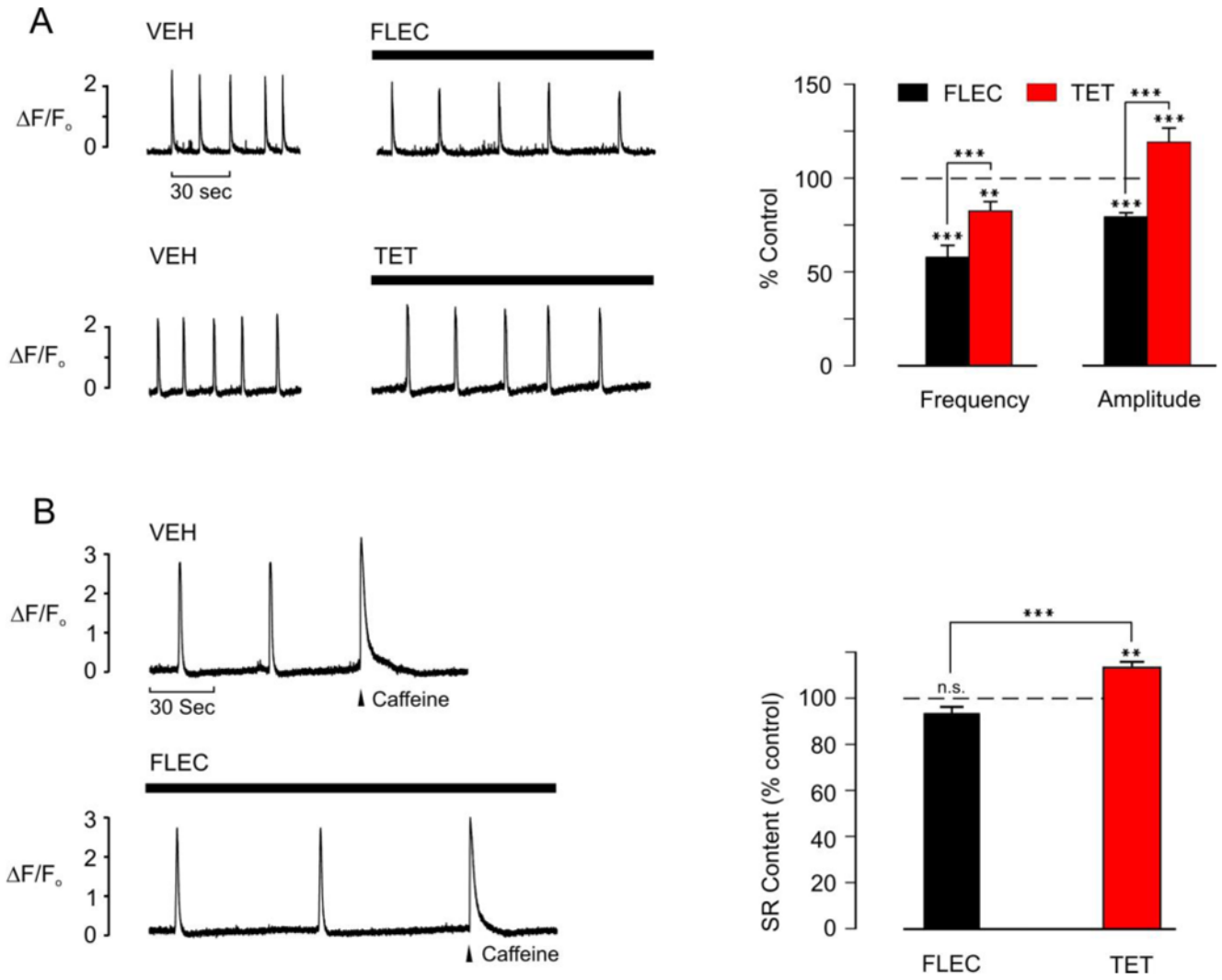

Fig. 3.

Effects of flecainide or tetracaine on Ca^{2+} waves and the SR Ca^{2+} content in permeabilized rat myocytes

(A) Representative integrated line-scan images (*left*) showing spontaneous Ca^{2+} waves in permeabilized ventricular myocytes during exposure to vehicle (VEH) and after 2 min exposure to either 25 μM flecainide (FLEC) or 50 μM tetracaine (TET). Average data (*right*) comparing the effects of flecainide and tetracaine on Ca^{2+} wave amplitude and frequency. FLEC n = 9, TET n = 7, **p<0.01, ***p<0.001.

(B) Representative integrated line scan images (*left*) showing spontaneous Ca^{2+} waves in permeabilized ventricular myocytes in the presence of vehicle or 2 min after introduction of 25 μM FLEC. In both cases, the wave frequency was monitored and 10 mM caffeine rapidly applied when a wave would otherwise have occurred (arrowhead). The amplitude of the caffeine-induce Ca^{2+} transient was used as an index of the SR Ca^{2+} content in each case. Average data comparing the effects of flecainide and tetracaine on the SR Ca^{2+} content under these conditions (*right*). FLEC n = 6, TET n = 6, **p<0.01, ***p<0.001, n.s. = not significant.

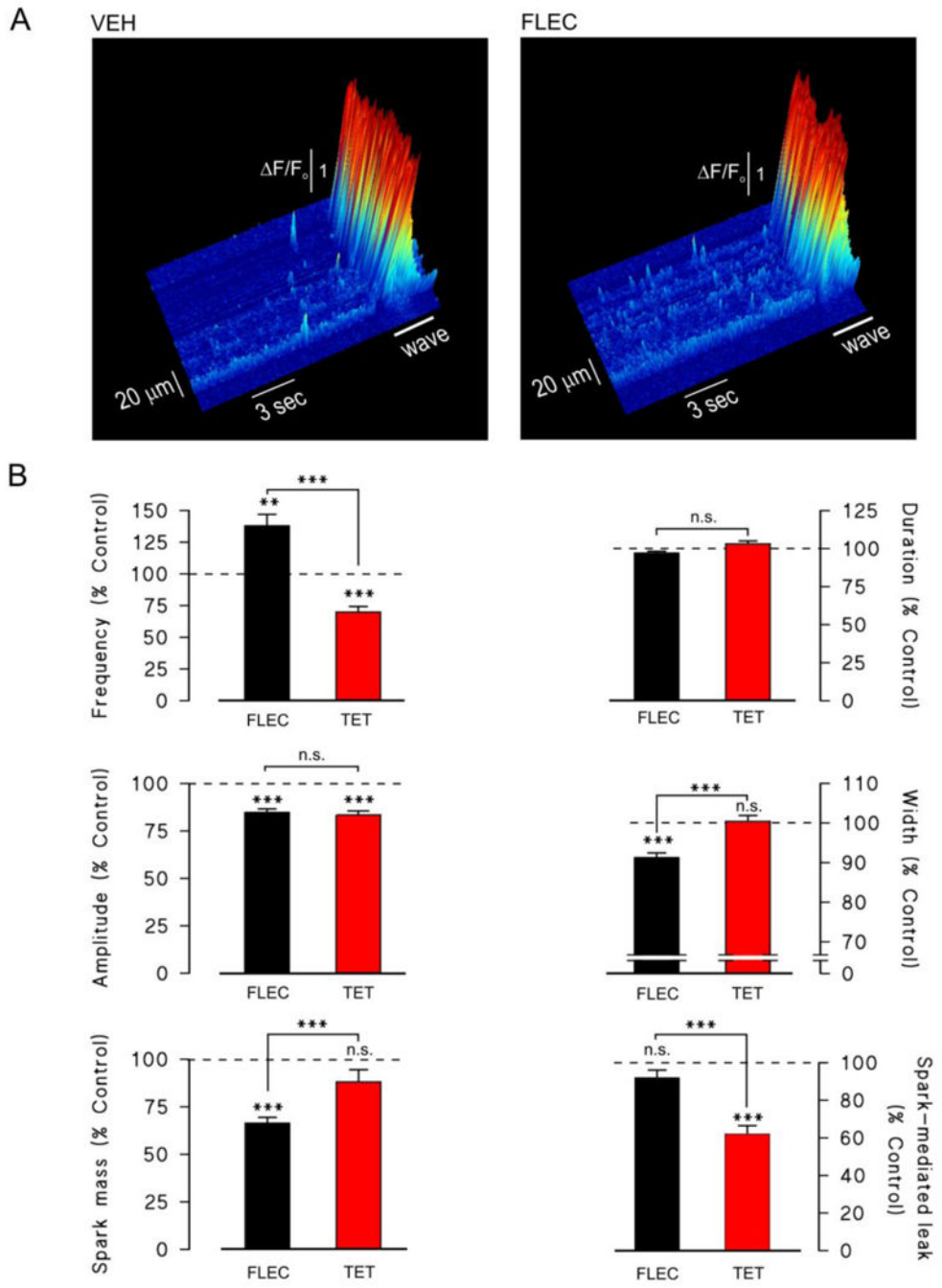

Fig. 4.

Effects of flecainide and tetracaine on spontaneous Ca^{2+} sparks in permeabilized myocytes

(A) Surface plots of raw line scan images obtained from a permeabilized rat ventricular myocyte during spontaneous Ca^{2+} wave generation, in the presence of vehicle (*left*) or after introduction flecainide (*right*). Note the increased spark frequency in the myocyte exposed to FLEC. The cell was bathed in weakly Ca^{2+} -buffered solutions (0.05 mM EGTA) approximating to the intracellular milieu, with a free [Ca^{2+}] of ~200 nM.

(B) Cumulative data showing the relative changes in Ca^{2+} spark frequency, amplitude, mass, duration and width following introduction of 25 μM flecainide or 50 μM tetracaine. Spark data

was obtained in more strongly Ca^{2+} buffered solutions (0.36 mM) to prevent Ca^{2+} wave propagation. **p<0.01, ***p<0.001, n.s. = not significant. 160-341 sparks from n = 25-33 myocytes per group.

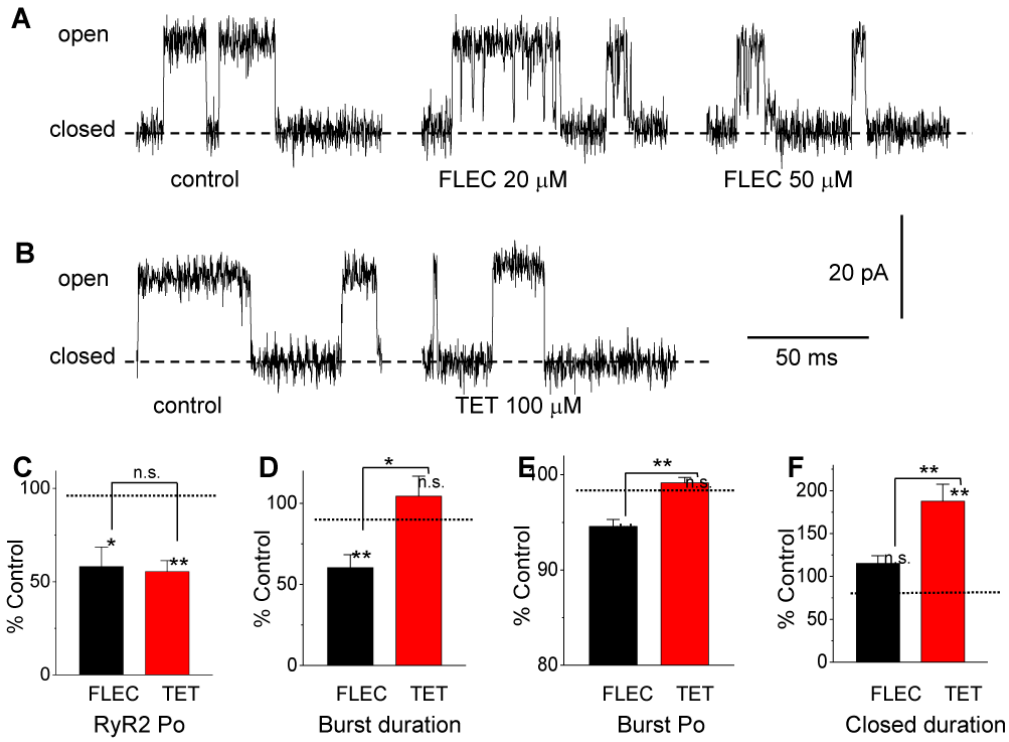

Fig. 5.

Flecainide and tetracaine block single RyR2 channels by different mechanisms.

(**A,B**) Records are representative examples of single channel activity of RyR2 in lipid bilayers. Control conditions were 1 mM luminal Ca^{2+} (*trans* bath), 0.1 μM cytoplasmc Ca^{2+} plus 2 mM ATP (*cis* bath). Bilayer potential was 40 mV (relative to *trans* bath as ground). Relatively high concentrations of flecainide (FLEC) and tetracaine (TET) were used to better illustrate their differential effect on RyR2 channel gating.

(**A**) Single experiment where flecainide was added to the cytoplasmic bath. Under control conditions, this channel had an open probability (P_o) of 0.25, mean open time (τ_o) of 83 ms and mean closed time (τ_c) of 228 ms. The full duration of typical closed periods is not seen on this time scale. Addition of flecainide introduced short (~ 1 ms) closures to a substate at ~20% of the full channel conductance which lead to bursts of short channel openings.

(**B**) Different experiment where tetracaine was added to the bath. Under control conditions, this channel had $P_o = 0.11$, $\tau_o = 33$ ms and $\tau_c = 237$ ms. Addition of 100 μM tetracaine increased τ_c to 913 ms but had little effect on τ_o (τ_o =27 ms).

(**C**) The effects of flecainide and tetracaine on burst parameters were compared at concentrations that reduced RyR2 Po by approximately 50% (10 μM and 50 μM, respectively).

(**D-F**) Comparison of average burst parameters derived from burst analyses of single channel recordings. The data are expressed as burst properties relative to control before addition of FLEC or TET (control conditions are given in methods). Note that FLEC inhibits RyR2 by reducing burst duration (**D**) and intraburst Po (**E**), whereas TET increased the interburst closed duration (**F**). *p<0.05, **p<0.01, n=10 per group

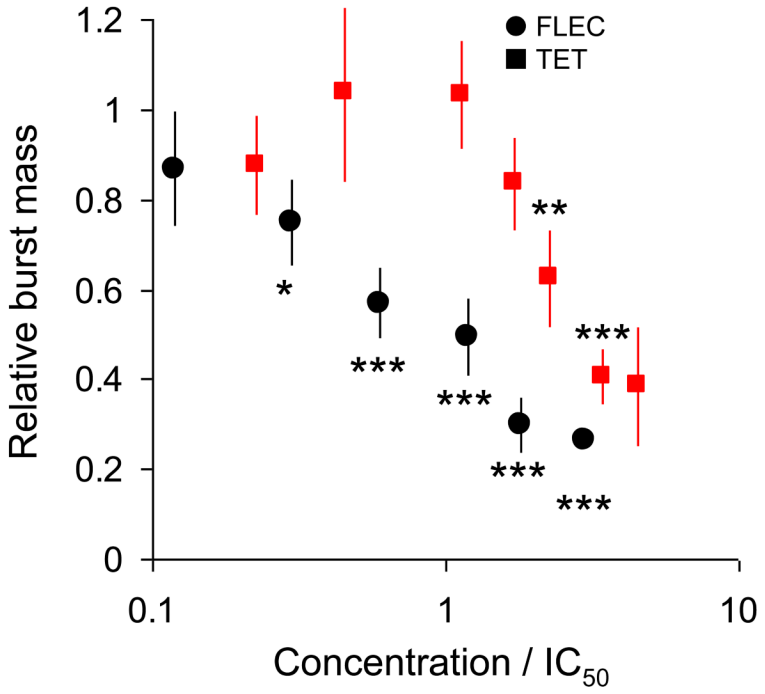

Fig. 6.

Flecainide and tetracaine block single RyR2 channels by different mechanisms. The concentration dependence of the effects of flecainide (●) and tetracaine (■) on the total RyR2 activity within bursts (burst mass). For ease of comparison, drug concentrations are expressed as a ratio of concentration and the IC_{50} values (flecainide 16 μM, tetracaine 44 μM). Burst mass was calculated from the product of the burst duration (Supplemental Fig. 1C) and the open probability within bursts (Supplemental Fig. 1D) normalized to values obtained in the absence of drug. *p<0.05, **p<0.01, ***p<0.001 compared to control by paired *t*-test.

Table 1

Ca^{2+} spark parameters in intact *Casq2-/-* myocytes

Parameters	**Vehicle (n = 87)**	**Flecainide (n = 78)**	**Tetracaine (n = 84)**	**ANOVA *P*-value**
Spark Frequency (Sparks·100μm^{-1}·s^{-1})	0.84±0.14	**1.44±0.16**$^{\#*}$	**0.48±0.10**$^{\#}$	**<0.001**
Amplitude ($\Delta F/F_0$)	0.89±0.04	**0.71±0.02**$^{\#*}$	0.89±0.02	**<0.001**
FWHM (μm)	3.35±0.05	**2.93±0.04**$^{\#*}$	**3.13±0.09**$^{\#}$	**<0.001**
FDHM (ms)	79.5±2.5	**67.3±1.8**$^{\#*}$	78.5±4.6.	**<0.001**
Full Width (μm)	5.52±0.12	**4.76±0.09**$^{\#*}$	5.31±0.16	**<0.001**
Full Duration (ms)	131±4.0	**110±3.0**$^{\#*}$	128±5.8	**<0.001**
Time to Peak (ms)	33.9±1.8	28.2±1.4	32.3±2.9	0.053
Maximum Steepness of Upstroke (Δ(F/Fo)/s)	57.5±2.1	53.1±1.7	58.8±3.1	0.266
Tau (ms)	91.6±4.9	113.0±22.1	87.9±6.0	0.583
Spark Mass ($(\Delta F/F_0)$ μm^3)	63.3±8.8	**31.1±4.0**$^{\#*}$	56.4±12.9	**<0.001**

All myocytes were bathed for 10 min in experimental solutions containing either vehicle (0.01% ethanol), flecainide (6 μM) or tetracaine (50 μM) prior to recording line-scans. FWHM: spark full width at half maximum amplitude, FDHM: spark duration at half maximum amplitude; data are mean ± S.E.M.; *p*-values of post-hoc *Student's T*-test:

$^{\#}$ $p < 0.05$ vs. vehicle;

* $p < 0.05$ flecainide vs. tetracaine; number of sparks analyzed per group: vehicle = 294, flecainide = 461, tetracaine = 155