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EFFICACY AND POTENCY OF CLASS I ANTIARRHYTHMIC DRUGS FOR SUPPRESSION OF Ca^{2+} WAVES IN PERMEABILIZED MYOCYTES LACKING CALSEQUESTRIN

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Abstract

Background— Ca^{2+} waves can trigger ventricular arrhythmias such as catecholaminergic-polymorphic ventricular tachycardia (CPVT). Drugs that prevent Ca^{2+} waves may have antiarrhythmic properties. Here, we use permeabilized ventricular myocytes from a CPVT mouse model lacking calsequestrin (casq2) to screen all clinically available class I antiarrhythmic drugs and selected other antiarrhythmic agents for activity against Ca^{2+} waves.

Methods and Results—Casq2^{-/-} myocytes were imaged in line-scan mode and the following Ca^{2+} wave parameters analyzed: wave incidence, amplitude, frequency, and propagation speed. IC₅₀ (potency) and maximum inhibition (efficacy) were calculated for each drug. Drugs fell into 3 distinct categories. Category 1 drugs (flecainide, R-propafenone) suppressed wave parameters with the highest potency (IC₅₀ < 10 μM) and efficacy (> 50% maximum wave inhibition). Category 2 drugs (encainide, quinidine, lidocaine, verapamil) had intermediate potency (IC₅₀ 20 μM –40 μM) and efficacy (20%–40% maximum wave inhibition). Category 3 drugs (procainamide, disopyramide, mexilitine, cibenzoline, ranolazine) had no significant effects on Ca^{2+} waves at the highest concentration tested (100 μM). Propafenone was stereoselective, with R-propafenone suppressing waves more potently than S-propafenone (IC₅₀: R-propafenone 2 $\pm$ 0.2 μM vs. S-propafenone 54 $\pm$ 18 μM). Both flecainide and R-propafenone decreased Ca^{2+} spark mass and converted propagated Ca^{2+} waves into non-propagated wavelets and frequent sparks, suggesting that reduction in spark mass, not spark frequency, was responsible for wave suppression.

Conclusions—Among all class I antiarrhythmic drugs, flecainide and R-propafenone inhibit Ca^{2+} waves with the highest potency and efficacy. Permeabilized casq2^{-/-} myocytes are a simple in-vitro assay for finding drugs with activity against Ca^{2+} waves.

Keywords

catecholaminergic polymorphic ventricular tachycardia; calsequestrin 2; cardiac ryanodine receptor (RyR2) Ca^{2+} release channel; class I antiarrhythmic drugs; flecainide; R-propafenone

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1. Introduction

Catecholaminergic-polymorphic ventricular tachycardia (CPVT) is a life-threatening ventricular arrhythmia that can occur in genetically predisposed individuals with structurally normal hearts when they are exposed to an increased catecholaminergic load such as during exercise or emotional stress [1]. Ca^{2+} waves are generally considered the underlying events that generate the delayed afterdepolarizations that trigger CPVT [2]. Ca^{2+} waves can occur due to (i) sarcoplasmic reticulum (SR) Ca^{2+} overload [3], or (ii) as a consequence of an alteration in the function of the cardiac Ca^{2+} release unit (CRU) [4]. The malfunction of the CRU has been attributed to either mutations in the gene that encodes the cardiac ryanodine receptor (RyR2) Ca^{2+} release channel, or mutations in cardiac calsequestrin (casq2) [5] [6], [7], [8]. The interaction between casq2 and junctin and triadin (two other SR proteins) assures clustering of casq2 at the junctional SR membrane facing the t-tubules [9]. Besides its function buffering the free Ca^{2+} inside the SR, single-channel data suggest that casq2 regulates the RyR2 open probability [10]. As a consequence of the occurrence of Ca^{2+} waves, the cytosolic Ca^{2+} concentration increases. This increase in the cytosolic Ca^{2+} forces the $\text{Na}^+/\text{Ca}^{2+}$ exchanger to work in the reverse mode, generating a secondary Na^+ current that depolarizes the sarcolemma (or plasma membrane) of the cardiac myocytes. If the magnitude of the depolarization is big enough, it will overcome the electrical threshold and trigger a propagated action potential. These membrane depolarizations are called delayed afterdepolarizations (DAD), and their occurrence has been proposed as the responsible mechanism for Ca^{2+} -triggered arrhythmia [2, 11]. Thus, in principle, the prevention of the occurrence of the Ca^{2+} waves should prevent the occurrence of the arrhythmia. Consistent with this idea, we recently reported that the class I antiarrhythmic drug flecainide suppressed Ca^{2+} waves by open channel RyR2 inhibition [12] and prevented CPVT in mice and humans [13].

Here we propose a simple *in-vitro* assay using permeabilized myocytes isolated from a mouse model of CPVT to screen drug potency and efficacy for suppressing Ca^{2+} waves. Compared to intact myocytes or voltage-clamped myocytes, the use of permeabilized myocytes provides important advantages: (1) Rapid access to the intracellular drug target, the RyR2 Ca^{2+} release channel complex; (2) Stable Ca^{2+} wave frequency and amplitude over extended periods of time; (3) Control of cytosolic concentration of Ca^{2+} (and thus, control of RyR2 channel gating by cytosolic Ca^{2+}), which excludes potential drug effects on sarcolemmal Ca^{2+} fluxes. For example, both verapamil [14] [15] and ranolazine [16] have been reported to inhibit Ca^{2+} waves, but it is not known if this effect involves regulation of SR Ca^{2+} release. We analyzed drug effects on wave incidence, amplitude, frequency, and propagation speed. An increase in any one of these four parameters independently increases the likelihood and amplitude of membrane depolarizations and therefore the arrhythmogenicity of Ca^{2+} waves [17], [18] [19]. Our study readily identified the two drugs, flecainide and R-propafenone, that have been reported to block single RyR2 channels in bilayers and prevent CPVT in mice and humans [13] [19]. This result suggests that our *in vitro* test may be useful as a tool to identify compounds with potential antiarrhythmic activity against ventricular arrhythmias caused by Ca^{2+} waves such as CPVT. Our data also suggest that propafenone (in particular its R-enantiomer) should be further evaluated as therapeutic agent in CPVT.

2. Methods

2.1. Experimental procedures and animal model of CPVT

The use of animals in this study was approved by the Animal Care and Use Committee of Vanderbilt University, USA, and performed in accordance with NIH guidelines. Here we used the casq2^{-/-} mice that display normal SR Ca^{2+} release and contractile function under

basal conditions, but consistently develop ventricular tachycardia during exercise or after catecholamine challenge [20].

Tetracaine, quinidine hydrochloride monohydrate, procainamide hydrochloride, disopyramide phosphate salt, lidocaine hydrochloride, mexiletine hydrochloride, encainide, flecainide acetate, propafenone hydrochloride, verapamil, cibenzoline and ranolazine dihydrochloride, as well as all the chemicals used to prepare the solutions (unless otherwise specified) were obtained from Sigma (St. Louis, MO). The stock solutions for all drugs tested were prepared using DMSO as solvent (final concentration 2.5 μ l DMSO/ml internal solution). In the case of propafenone, (+)-S-propafenone and (–)-R-propafenone were separated on a ChiralPak AD column (25 $\times$ 0.46 cm, Chiral Technologies, Exton, PA) using hexane and 2-propanol containing 0.4% diethylamine. The flow rate was 1 ml and UV absorption was monitored at 247 nm. The purity of these two enantiomers was greater than 99%. The internal solution used as a control solution contained 2.5 μ l DMSO/ml internal solution (VEH solution).

2.2. Isolation of ventricular myocytes

Single ventricular myocytes from 14 to 20 weeks-old *casq2*^{–/–} mice were isolated by enzymatic digestion applying a modified collagenase/protease method as previously described [20]. Briefly, the mice were anesthetized with isoflurane. Immediately after, the heart was removed from the chest and the aorta was cannulated and placed onto a Langendorff perfusion system. The hearts were retrograde perfused (33.5–34°C) at a constant flow rate with modified tyrode solution for 3 minutes. Next, the hearts were perfused with the enzymatic solution containing collagenase (from Worthington) and protease (from Sigma) for 7–8 min. Once the perfusion with enzyme solution was finished, the hearts were removed from the cannula and placed in a Petri dish with 0.2 mM CaCl₂ BSA/tyrode solution. Atria and other conjunctive tissues were discarded, and then the ventricles were minced into small pieces with scissors. The release of myocytes was achieved by gently pipetting with a transfer pipette until most of the tissue has disintegrated. The cells were washed twice by gravity sedimentation for 20 min in 0.2 mM CaCl₂ tyrode solution. Finally, the supernatant was removed and the cells were resuspended in 0.6 mM Ca₂Cl DMEM solution (Dulbecco's Modification of Eagle's Medium).

2.3. Experiments in permeabilized ventricular myocytes

An aliquot of about 200 μ l of the solution containing the isolated myocytes was placed in a laminin-coated chamber and allowed to settle down for 6 min before starting the permeabilization. In these chambers, fluid is exchanged in bulk for drug application. Once myocytes settled down at the bottom of the chamber, they were first exposed to a relaxing solution containing (in mM): ethylene glycol-bis (2-aminoethylester)-N, N, N', N'-tetraacetic acid (EGTA) 0.1, HEPES 10, K-aspartate 150, MgCl₂ 0.25, and Adenosine TriPhosphate di-Na⁺ (di-Na⁺ ATP) 5. After 30 s, the supernatant was replaced with the internal solution containing saponin (40 μ g/ml). The cells were exposed to the saponin solution for 1 min. The saponin solution was removed and replaced by control internal solution, which contained (in mM): K-aspartate 100, KCl 15, KH₂PO₄ 5, CaCl₂ 0.04 – 0.06, MgCl₂ 0.75, Dextran (40,000) 8 %, HEPES 10, MgATP 5, phosphocreatine DiNa⁺ 10, Creatine phosphokinase 10 U/ml, Glutathione (reduced) 10, Fluo 4 pentapotassium salt 0.02. Low cytosolic buffering conditions (EGTA 0.05 mM in internal solution) were used to allow spontaneous propagated Ca²⁺ waves, as previously reported [21]. At a concentration of Ca²⁺ of 0.04 mM in the internal solution (estimated free [Ca²⁺] 0.5 μ M (Maxchelator)), only slightly more than half (62%) of the *casq2*^{–/–} myocytes exhibited stables Ca²⁺ waves. In order to maximize the chance to detect drug effects on propagated Ca²⁺ waves, we increased [Ca²⁺] to 0.06 mM (estimated free [Ca²⁺] 3.9 μ M (Maxchelator)). Under these conditions,

almost all of myocytes (~90%) exhibited spontaneous Ca^{2+} waves with a stable Ca^{2+} wave frequency and amplitude over extended periods of time (> 20 min, Supplemental Fig. 1). In all cases, the pH of the internal solution was corrected to 7.2 with KOH. All the experiments in permeabilized myocytes were performed at room temperature (22-24° C).

To measure Ca^{2+} sparks, the concentration of EGTA in the internal solution was increased to 0.4 mM (to avoid the generation of Ca^{2+} waves). The total concentration of Ca^{2+} used in these experiments was 0.06 mM. Under these high-buffered conditions, the estimated free Ca^{2+} concentration was about 30 nM (Maxchelator). A subset of cells was rapidly exposed to 10 mM caffeine to assess the SR Ca^{2+} content under each condition. Amplitudes of caffeine-induced Ca^{2+} transients were used as estimates of SR Ca^{2+} content.

2.4. Confocal imaging

After permeabilization, the myocytes did not exhibit any obvious changes in their shape compared to intact myocytes (Fig. 1A). As an internal control, we measured the length and the width of *casq2*^{-/-} myocytes before and after the cells were permeabilized, and based on that data calculated the cell surface. We did not find any statistically significant differences in the length of the myocytes before and after permeabilization (128.2±2.8 µm in intact myocytes vs 129.9±2.4 µm in permeabilized myocytes). Similarly, there was no statistically significant difference in the width between both groups (22.2±0.7 µm in intact myocytes vs. 23.8±0.7 µm in permeabilized myocytes). Intact *casq2*^{-/-} myocytes, n=65 cells; permeabilized *casq2*^{-/-} myocytes, n=53 cells.

The permeabilized cells were imaged with an LSM 510 Zeiss inverted microscope and a 40x oil immersion objective lens (Nikon, Tokyo, Japan). Intracellular fluo-4 was excited at 488 nm with a krypton/argon laser. The fluorescent emission was collected through a long-pass filter (>515 nm). All images were acquired digitally in line-scan mode with 0.2 µm and 0.2 ms per pixel resolution. We only collected data from cells that showed the classic brick-shape of a healthy ventricular myocyte and that were separated from each other, as illustrated in Fig. 1B. Since the effect of drugs on Ca^{2+} wave parameters appeared to be relatively stable after 10 min (Supplemental Fig. 1), all measurements were taken after incubation for 10 min with internal solutions containing either VEH or study drug. We then identified two populations of cells: cells that exhibited repetitive and continuous Ca^{2+} wave fronts across the full width of the myocytes (“*waving*” myocytes), and cells that were not waving any more, but instead showed only Ca^{2+} sparks (“*sparking*” myocytes). For each drug concentration, we examined between 7 and 10 microscope fields. As depicted in Fig. 1B, the fields were selected scanning the chambers following the Greek-shaped scanning pattern (square-shape red line). This is a standard procedure that is normally used to study the cells on blood smears and avoid repeating fields that already have been examined [22], [23]. Wave incidence was calculated as the fraction of cells waving divided by the total number of brick-shaped myocytes per field.

2.5. Data analysis

In all cases, fluorescence images were analyzed using ImageJ, the public domain NIH Image program (developed at NIH and available on the internet at <http://rsb.info.nih.gov/niH-image>). Figure 2 shows the process of analyzing Ca^{2+} waves. A light-transmitted image of a permeabilized cardiac myocyte (1) is shown lying flat on the cover slip (left side of Fig. 2). The scan line was placed parallel to the longitudinal (main) axis of the myocyte. A line-scan (LS) (2) and its averaged-space record (ASR) (3) are shown on the right side. LS were obtained only from the population of waving cells. For the purpose of the wave analysis in the present work, a propagated Ca^{2+} wave was defined as a continuous wave front in the LS image visualized as a robust fluorescent line that propagates

across the full width and along the entire length of the myocyte without breaking down into sparks. Wave amplitude, frequency, and propagation speed were calculated for each drug concentration and concentration-response curves constructed for each wave parameter. The corresponding IC₅₀ was obtained by fitting the curves to a Boltzmann function using OriginLab non-linear fitting software. In general, one parameter was initially held fixed for the initial fitting, but after that all the parameters were allowed to vary freely. For the purpose of comparing different drugs, *efficacy* of a drug was defined as the percentage of maximal suppression or decrease in the wave parameter at 100 μ M, which was the highest concentration of all drugs tested. All wave parameters presented in this work (wave incidence, amplitude, frequency, and speed of wave propagation) have been used previously as independent predictors of arrhythmogenic potential of Ca²⁺ waves, that is, they can be used to predict the likelihood of Ca²⁺ waves to generate a delayed membrane depolarization (DAD) and triggered beats [17] [18] [19]. All parameter values were normalized to parameter values obtained from cells exposed for 10 min to VEH (DMSO).

In a separate set of experiments, Ca²⁺ sparks were analyzed. The automated detection of Ca²⁺ sparks and the measurement of temporal and spatial spark properties was carried out using the “SparkMaster” plug-in for ImageJ. The detection criteria were set at 3.8, that is, the threshold for the detection of events was 3.8 times the standard deviation of the background noise divided by the mean [24]. Spark mass was calculated as amplitude X 1.206 X FWHM³ [25].

2.6. Statistical analysis

The comparisons of wave incidence between the experimental and control groups were done using Fisher exact test. All other parameters were compared using non-paired Student *t*-test. Results were considered statistically significant if the p-value was less than 0.05.

3. Results

3.1. Effect of drugs on Ca²⁺ wave incidence

To test the hypothesis that class I antiarrhythmic agents inhibit Ca²⁺ waves by direct action on SR Ca²⁺ cycling, we screened all FDA-approved class I antiarrhythmic drugs using our permeabilized casq2^{-/-} myocyte assay. We also included verapamil (Ca²⁺ channel blocker, class IV antiarrhythmic agent) and ranolazine (a novel anti-anginal agent). Both agents have been shown to inhibit Ca²⁺ waves in intact myocytes and have been proposed as alternatives for the management of CPVT [14] [15] [16]. Figure 3A shows representative LS obtained at 1, 5 and 15 min after the cells were exposed to internal solution containing either VEH or study drug. Cells exposed to VEH exhibited robust Ca²⁺ waves of stable frequency and amplitude. In contrast, exposure to flecainide caused a rapid and progressive break-up of the Ca²⁺ waves in some myocytes (Fig. 3A), and reduced wave frequency in the remaining myocytes (Supplemental Fig. 1). As illustrated in Fig. 3A, the highly organized, propagated Ca²⁺ waves were replaced by non-propagated wavelets and Ca²⁺ sparks. The effect of flecainide was in stark contrast to that of another RyR2 channel blocker, tetracaine, which had the opposite effect. Tetracaine stabilized the structure of the Ca²⁺ waves and increased their amplitude. We previously showed that tetracaine primarily blocks RyR2 channels in their closed state, whereas flecainide is an open state channel blocker [12]. Consistent with the idea that open state RyR2 block is important for Ca²⁺ wave suppression, the R-enantiomere of propafenone, which is an open channel blocker analogous to flecainide [26], also rapidly suppressed waves by either breaking them down into sparks (depicted in the last LS sequence of Fig. 3A) or reducing the frequency of waves (Supplemental Fig. 1). We next obtained concentration-response curves for every drug that significantly changed the wave incidence after 10 min exposure to a concentration of 100 μ M (Fig. 3B). With respect to

wave incidence, R-propafenone and flecainide showed the highest potency (= lowest IC_{50}) and efficacy (= %inhibition with 100 μ M) for suppressing Ca^{2+} waves (Fig. 3B and Table 1). Even though these two drugs showed similar efficacies for suppressing propagated waves (~70%), R-propafenone was about 6 times more potent than flecainide ($12.8 \pm 0.6 \mu$ M and $2.0 \pm 0.6 \mu$ M respectively). In contrast, S-propafenone was less potent and had a lower efficacy in terms of wave suppression than flecainide and R-propafenone. Racemic propafenone showed an intermediate potency and efficacy (Fig. 3B and Table 1). Encainide, quinidine, lidocaine and verapamil suppressed Ca^{2+} waves only at concentrations above 20 μ M. All other drugs tested had no significant effect on Ca^{2+} wave incidence at the highest concentration tested (100 μ M). Interestingly, even at 100 μ M (highest concentration tested), neither flecainide nor R-propafenone was able to completely suppress waves in all cells, resulting in a maximum efficacy of approximately 70% (Table 1). Finally, tetracaine was the only drug that significantly increased Ca^{2+} wave incidence (by ~30% relative to VEH).

To gain insight what determines whether cells exhibit continuous Ca^{2+} waves or only sparks, we compared the SR Ca^{2+} load in waving cells and in non-waving “sparking” cells under the same buffering conditions ([EGTA]= 0.05 mM). The majority of cells (~90%) were waving under these experimental conditions. There was no statistically significant difference between waving cells and sparking cells in SR Ca^{2+} content estimated by amplitude of the caffeine-induced Ca^{2+} transients ($1.5 \pm 0.07 F/F_0$ (n=18) vs $1.41 \pm 0.08 F/F_0$ (n=5) respectively). Thus, a change in SR load does not appear to determine whether a particular myocyte waves or sparks. This result is consistent with our data showing that both flecainide and propafenone suppress Ca^{2+} waves without changing SR load [26].

3.2. Potency and efficacy of reducing amplitude, frequency and propagation speed of Ca^{2+} waves

Since the majority of cells continued to have full-fledged Ca^{2+} waves after 10 min drug exposure, we next analyzed the effects of the drugs on three Ca^{2+} wave parameters that have been implicated as independent predictors of arrhythmogenicity: wave amplitude, frequency and propagation speed. Supplemental Fig. 1A shows examples of myocytes that continued to exhibit Ca^{2+} waves in presence of flecainide and propafenone. Both drugs significantly reduced the frequency of Ca^{2+} waves, with a maximum effect reached after 10-15 min of drug exposure (Supplemental Fig. 1B). Full concentration response curves were obtained for each wave parameter (Fig. 4) and IC_{50} values calculated for each drug that showed a significant effect at 100 μ M. The results are summarized in Table 1. In general, the effect of the drugs on the individual wave parameters followed the effect of these drugs on the wave incidence (Table 1). To better illustrate this result, we averaged the IC_{50} and efficacy values of the four wave parameters for each drug (Fig. 5). Drugs fell into three distinct categories. Category 1 drugs, which included flecainide and R-propafenone, suppressed wave parameters with the highest potency (lowest IC_{50} concentrations [$<10 \mu$ M]) and efficacy (>50% maximum suppression, Fig. 5). Category 2 drugs (which included encainide, lidocaine, quinidine and verapamil) had measurable, but rather modest effects on wave amplitude, frequency, and speed of propagation (Table 1, and Figs. 4 & 5). This group of drugs exhibited intermediate potency ($IC_{50} = 20 - 40 \mu$ M) and efficacy (20 - 40 % maximum wave inhibition). Finally, category 3 drugs (which included procainamide, disopyramide, mexiletine, cibenzoline, and ranolazine) had no significant effects on wave parameters at the highest concentration tested (100 μ M) (Table 1, data not depicted in Figs. 4 & 5). Similarly to its effect on wave incidence, the effect of propafenone on the other wave parameters was stereoselective, with R-propafenone being more potent than S-propafenone and propafenone racemate. This result is consistent with propafenone's stereoselective inhibition of RyR2 channels in artificial lipid bilayers [26]. Tetracaine also reduced wave frequency and propagation speed, but increased the wave amplitude. The

latter effect would cause a more pronounced membrane depolarization and increase the likelihood of triggering a premature beat [2]. Taken together, the net effect of tetracaine may be a proarrhythmogenic one, since it increased wave incidence and amplitude by ~30 % relative to VEH.

3.3. Reduction of spark mass correlates with suppression of Ca^{2+} waves

In order to gain mechanistic insights as to explain why open-channel blockers such as flecainide and R-propafenone were so effective in breaking up Ca^{2+} waves in permeabilized myocytes, we next measured their effect on Ca^{2+} sparks (Fig. 6). In agreement with results previously obtained in intact myocytes [12], flecainide increased the spark frequency but decreased the spark mass (Fig. 6). R-propafenone had a similar effect on Ca^{2+} sparks as flecainide, that is, it significantly increases the spark frequency and decreases spark mass (Fig. 6). Neither flecainide nor R-propafenone changed SR Ca^{2+} content. On the other hand, even though tetracaine decreased spark frequency, it increased both spark mass and SR Ca^{2+} content. Since tetracaine did not prevent the occurrence of Ca^{2+} waves but rather increased it (Fig. 3A, 3B), these results suggest that reduction of spark mass is critically important for breaking up Ca^{2+} waves.

4. Discussion

4.1. Flecainide and R-propafenone act as “ Ca^{2+} wave busters”

Our data show that flecainide and R-propafenone were the only drugs that broke up propagated Ca^{2+} waves into non-propagated Ca^{2+} release events at low-micromolar concentrations. The suppression of the waves revealed Ca^{2+} sparks as the underlying local Ca^{2+} release events within the waves [18]. This result is consistent with flecainide and R-propafenone efficacy for preventing Ca^{2+} waves in intact myocytes and CPVT in mice and humans [12] [13] [26].

Propagated Ca^{2+} waves are the bulk response that emerges when spontaneous Ca^{2+} sparks trigger additional sparks due to the activation of neighboring RyR2 clusters acting in a regenerative manner. Even though in the cell Ca^{2+} sparks do not normally propagate, the increased probability of spatial recruitment (with the generation of a wave) could reflect changes in the Ca^{2+} spark/s that trigger/s the subsequent massive release. Cheng et al. [18] previously reported that when they increased the concentration of Ca^{2+} in the bathing solution from 1 to 10 mM, spark frequency, amplitude, and full-width at half-maximum (FWHM) were significantly increased. At the same time, there was an increase in the wave incidence from ~20 % of cells waving at 1 mM Ca^{2+} up to ~90 % when the cells were exposed to a solution containing 10 mM Ca^{2+} . This group also reported that in the majority of the waving cells Ca^{2+} sparks occurred near the site of wave initiation. In ~65 % of the cases, Ca^{2+} sparks were colocalized (within 5 μm and 50 ms) with the site of wave initiation. This colocalization suggests that Ca^{2+} sparks initiate or “trigger” the propagated Ca^{2+} waves. The increase in spark amplitude and FWHM in parallel to the increase in the wave incidence suggest that larger and bigger sparks tend to favor the occurrence of the waves [18].

In CPVT, *in-vitro* experiments have shown that there is an increase in the open probability of the RyR2, resulting in a hyperactive or “leaky” RyR2 that increases diastolic cell Ca^{2+} and the risk to arrhythmias [27, 28] [29]. We previously quantified the Ca^{2+} sparks as spark mass, and used it as an estimator of the magnitude of the local release of Ca^{2+} [12]. The spark mass is directly proportional to the spark amplitude and FWHM [25]. Flecainide decreased the spark mass by blocking RyR2 channels in the open state without any significant effect on the Ca^{2+} content of the SR [12]. In agreement with that, in the present

study only flecainide and R-propafenone decreased the spark mass without any significant effect on the SR Ca^{2+} content (Fig. 6B). R-propafenone seems to have a flecainide-like effect on sparks, since it also increased the spark frequency and decreased the spark mass, without any significant effect on the SR Ca^{2+} content. Due to the saltatory nature of the propagation/activation of the RyR2 clusters in the SR, a decreased spark mass should block the spread of the activation between neighboring RyR2 clusters, and prevent the wave. In this context, the modulation of the spark mass may be a predictor of therapeutic efficacy for blocking the production of Ca^{2+} waves and thus, the occurrence of the arrhythmia.

Several studies have examined the behavior of Ca^{2+} waves in response to experimental RyR2 modulators, mostly using tetracaine as prototype RyR2 channel inhibitor [30-32] [33]. We found here that tetracaine's effect on Ca^{2+} waves was very different from that of all other drugs tested. Tetracaine was the only drug that increased Ca^{2+} wave incidence and amplitude, whereas all other drugs had the opposite effect (Figs. 3 and 4). This result suggests that tetracaine may not be a "typical" RyR2 channel inhibitor and raises interesting questions regarding the validity of experiments that use tetracaine as a tool to study the effect of RyR2 channel inhibition on myocyte Ca^{2+} handling.

Loughrey et al. (2007) tested K201, a putative RyR2 channel "stabilizer" [34] [35], in normal adult rabbit ventricular cardiomyocytes permeabilized with β -escin. At 1 μM K201 reduced the frequency and velocity of the waves with no change in SR Ca^{2+} content. It also reduced Ca^{2+} spark amplitude and frequency. At 3 μM K201 completely abolished Ca^{2+} waves and reduced the SR Ca^{2+} content (to ~73%). Assays specific to SR Ca^{2+} -ATPase and RyR2 activity indicated that K201 inhibited both SR Ca^{2+} uptake and release. When comparing these results with data presented here, the main difference is that K201 decreases both the amplitude and frequency of sparks. At the highest concentration tested (3 μM) there is an additional effect on the SR Ca^{2+} content, which is significantly decreased by K201 [36]. In comparison, both flecainide and propafenone increased the spark frequency and decreased the spark mass, without affecting the SR Ca^{2+} content.

As previously proposed by us [12], the paradoxical effect of flecainide on spark frequency (and in the present study, the effect of flecainide-like drugs like R-propafenone) may be explained by the fact that flecainide acts mainly as an open channel blocker, with no effects on the RyR2 closed time. Basically, flecainide decreases the open times and the conductance of the RyR2 by causing brief closures to a subconductance state. This, in turn, decreases the burst mass, which likely explains the reduced spark mass in both intact and permeabilized myocytes. With respect to the effect on spark frequency, we previously reported that flecainide decrease both open and closed times of RyR2 channels activated by high cytosolic Ca^{2+} (100 μM), which could explain the dual effect of reduced spark mass and increased spark frequency in permeabilized myocytes [13].

Alternatively, the increased spark frequency could be explained by the effects of flecainide on spark mass and the consequent smaller magnitude of local Ca^{2+} depletion inside the SR: The amount of Ca^{2+} in the SR plays a central role in regulating normal RyR2 gating. The decrement in luminal Ca^{2+} induces the closure of the RyRs that terminates Ca^{2+} sparks under normal conditions [18] [37] [38] [39]. The smaller reductions in the local luminal SR Ca^{2+} observed with flecainide (decrease in spark mass) would favor RyR2 reopening and thus increasing spark frequency. This is analogous to the cytosolic control of the open probability of RyR2, where the local concentration of Ca^{2+} in the area immediately surrounding the RyR2 cluster has a great influence in the regulation of the open probability of the RyR2 individual channels [40].

4.2. Utility of permeabilized casq2^{-/-} myocytes for screening activity of compounds against Ca²⁺ waves

Due to the key role of catecholamines in triggering the arrhythmia, the standard therapy for CPVT are beta-blockers [41]. Unfortunately, beta blockers are not completely effective, with inadequate treatment responses in up to 50 % of CPVT patients [8]. At the same time, the study of alternative agents to treat arrhythmias like CPVT, as well as the development of new antiarrhythmic medications, is hindered due to the lack of predictive *in-vitro* assays for testing new drugs or compounds. In the present study we propose an *in-vitro* assay using permeabilized myocytes isolated from a mouse model of CPVT as a rapid and simple tool to screen drug potency and efficacy for suppressing Ca²⁺ waves. This assay allows constructing concentration-response relationships that can be used to calculate the potency (IC₅₀) and efficacy of the drugs tested, which may be relevant for predicting their antiarrhythmic action *in-vivo*. For example, only flecainide and R-propafenone prevented Ca²⁺ waves with low micromolar IC₅₀ concentrations that are close to serum concentrations achieved during clinical therapy with those agents [42].

Verapamil, a L-type Ca²⁺ current (I_{Ca,L}) blocker and class IV antiarrhythmic agent, has been reported to also interact with the skeletal ryanodine receptor (RyR1) and decreases the binding of [3H]ryanodine with an IC₅₀ of ~8 μM [43]. Based in part on its block of skeletal RyR1, Alcalai et al. tested verapamil in casq2 mutant mouse models that have been shown to reproduce the clinical CPVT phenotype [15]. Intraperitoneal administration of verapamil almost completely prevented arrhythmias *in vivo*. In intact myocytes, verapamil (1 μM) prevented the rise of diastolic Ca²⁺ caused by epinephrine, increased the amplitude of the Ca²⁺ transients, prevented spontaneous Ca²⁺ releases, and restored the Ca²⁺ in the SR. In our assay we used permeabilized myocytes to test whether direct regulation of SR Ca²⁺ release contributes to verapamil's effect in intact CPVT myocytes. Under this experimental condition, the cytosolic Ca²⁺ concentration is clamped and any contribution of drug effects on transsarcolemmal Ca²⁺ fluxes (i.e., L-type Ca²⁺ channels) is eliminated. Even though verapamil exerted a moderate effect on Ca²⁺ waves (Fig. 5), this occurred at 30-fold higher concentrations than the concentration of 1 μM studied by Alcalai et al. The upper limit of therapeutic range of verapamil in human serum is even lower, approximately 0.3 μM [42, 44]). Thus, the protective effect of verapamil in the CPVT mouse model is probably not caused by RyR2 inhibition, but more likely by inhibiting L-type Ca²⁺ channels and trans-sarcolemmal Ca²⁺ flux.

Ranolazine is a novel anti-anginal agent and has been proposed to have additional antiarrhythmic properties and inhibit Ca²⁺ waves [16]. Ranolazine was originally included in the class I antiarrhythmic group, but it also has mechanisms shared with class III and IV agents. Despite its many electrophysiological effects, ranolazine's main antiarrhythmic action has been attributed to its inhibition of late I_{Na} and the changes in the cellular Na⁺ content. In our assay, ranolazine did not show any significant effect on Ca²⁺ waves (Table 1). Similar to verapamil, our results suggest that ranolazine's antiarrhythmic activity does not involve direct regulation of SR Ca²⁺ release.

The availability of the *in-vitro* assay presented here offers other potential uses. Caspi et al. [45] have proposed that human embryonic stem-cells cardiomyocytes assessed with single-cell electrophysiology and microelectrode array mapping can serve as valid models for electrophysiological drug screening. Thus, our assay could be used to rapidly screen existing drugs or new compounds not only in myocytes from experimental models of CPVT, but also in myocytes derived from induced pluripotent stem (iPS) cells that can be harvested from CPVT patients [46].

Several limitations should be considered. Tissue concentrations of a drug in the target organs are often poorly correlated with the serum concentration, and could be much higher or lower. Furthermore, we did not test concentrations above 100 μM , because finding wave inhibition at higher concentrations ($>100 \mu\text{M}$) would have no clinical relevance for drug action. While this approach drastically reduces the number of experiments required per drug, the estimates of drug IC_{50} and efficacy reported by us are necessarily arbitrary and based on the highest concentration tested of 100 μM . In addition, our *in-vitro* assay is not necessarily specific and would also identify compounds that inhibit Ca^{2+} waves independent of RyR2 action (e.g., SR Ca^{2+} depletion by inhibition of SERCA). Those compounds will reduce cardiac contractility and would unlikely be of clinical value. Finally, we only tested drugs in myocytes lacking calsequestrin. Drug potency and efficacy might be different in other CPVT models and will have to be tested in future experiments. Nevertheless, we consider it likely that the results can be extrapolated to RyR2 mutations or potentially to other types of Ca^{2+} triggered arrhythmias (e.g. digitalis overdose) since Ca^{2+} waves are the fundamental mechanism in all cases. The clinical efficacy of flecainide in CPVT patients with RyR2 mutations further supports this concept [47].

5. Conclusions

Here, we used an *in-vitro* assay to test the effects of all class I antiarrhythmic drugs on permeabilized myocytes from a murine *casq2*^{-/-} model of CPVT. Among all drugs tested, flecainide and R-propafenone inhibit arrhythmogenic Ca^{2+} waves with the highest potency and efficacy. This finding is consistent with their *in-vivo* efficacy in mice. Based on our results, propafenone (mainly its R-enantiomer) should be further evaluated as target-specific therapeutic agent in CPVT. Furthermore, the spark data support the hypothesis that a reduction in spark mass, not spark frequency, predicts the antiarrhythmic activity of drugs that inhibit RyR2 channels. Our results suggest the possibility of using permeabilized ventricular myocytes from a CPVT mouse model to discover new antiarrhythmic drugs. A similar approach could be used on induced pluripotent stem (iPS) cells from patients with CPVT, to provide personalized drug therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Permeabilized ventricular myocytes from CPVT mice were used as drug screening assay
2. Potency and efficacy of Ca^{2+} wave inhibition were analyzed for each drug
3. Flecainide and R-propafenone had highest potency and efficacy of wave inhibition
4. Drug screening assay predicts *in vivo* antiarrhythmic efficacy in CPVT mice
5. Assay may be useful for testing drug activity against Ca^{2+} triggered arrhythmias

Figure 1.

Panel A: Light transmitted images of a *casq2*^{-/-} cardiac ventricular myocyte before (1) and after (2) permeabilization. Panel B. Schematic representation of a chamber used to image the myocytes and the pattern followed to select the fields for calculating wave incidence (7 - 10 fields per drug concentration). Chambers are rectangular of approximately 0.5 ml volume. Fluid is exchanged in bulk for drug application. There is no continuous flow. The microscopic fields were selected following a Greek-scanning pattern (depicted with a red squared-line) to avoid repeating fields that already have been examined. The green circle is zoom-in image of a microscopic field at a low magnification (20x) and shows permeabilized cardiac myocytes that are waving. Yellow dashed rectangles outline individual myocytes. In each cell, there are two types of prominent fluorescent signals, the repetitive Ca^{2+} wave propagating in linear wave fronts along the cell, and the somewhat brighter, but much smaller and static signals that represent the nuclei (arrow).

Figure 2.

Ca^{2+} wave analysis. Shown is a light-transmitted image of a permeabilized cardiac myocyte lying flat on the cover slip (left side of the panel, 1). The source of a Ca^{2+} wave is also represented with concentric circles (in yellow and orange), as well as the line used to obtain the corresponding line-scans (LS, 2). After we obtained the line scans (LS), a rectangular region of interest (ROI) is selected (red). ROI was always $\sim 3 \mu\text{m}$ x full length of the LS. LS and the averaged-space record (ASR, 3) are depicted on the right side of the panel. These records were used to measure the following wave parameters: frequency, amplitude, and propagation speed along the myocyte.

Figure 3.

Panel A: Only flecainide and R-propafenone rapidly suppress Ca^{2+} waves. Representative line-scans that show Ca^{2+} waves in permeabilized $\text{casq2}^{-/-}$ myocytes under control conditions (vehicle, VEH), and after switching to either VEH, flecainide (FLEC) 25 μM , tetracaine (TET) 50 μM , or R-propafenone (R-PROP) 25 μM , at 1, 5, and 15 min after the change was made (indicated by the red arrow). Panel B: Concentration-response curves (CRC) of wave incidence as a function of the drug concentration (in μM) for all drugs with $\text{IC}_{50} < 100 \mu\text{M}$. The mean values for each concentration are represented. $n=25-30$ cells / condition tested.

Figure 4. Concentration-response curves of wave frequency, wave amplitude, and wave propagation speed as a function of the drug concentration (μM) for all drugs with $\text{IC}_{50} < 100 \mu\text{M}$. Mean $\pm$ SEM is depicted for each concentration and each parameter. $n=15-20$ cells / condition tested.

Figure 5.

Average drug potency (IC₅₀) and efficacy of Ca²⁺ wave suppression. Data are mean and SEM calculated by averaging the IC₅₀ and efficacy values of the four wave parameters (incidence, amplitude, frequency and propagation speed) listed in Table 1. Category 1 drugs (flecainide and R-propafenone) suppressed wave parameters with the highest potency (IC₅₀ < 10 μM) and efficacy (> 50% maximum wave inhibition). Category 2 drugs (S-propafenone, encainide, quinidine, lidocaine and verapamil) had intermediate potency and efficacy. The vertical dashed lines represent the cut-off values between the two categories. Category 3 drugs had no significant effect at 100 μM and are not shown.

Figure 6.

Panel A: Examples of line-scans of Ca^{2+} sparks obtained in $\text{casq2}^{-/-}$ myocytes in control conditions (VEH), and in the presence of flecainide (FLEC) 25 μM , tetracaine (TET) 50 μM , and R-propafenone (R-PROP) 10 μM . Panel B: Comparison of average spark mass, spark frequency, and SR Ca^{2+} content for FLEC 25 μM , TET 50 μM , and R-PROP 10 μM (% VEH). $n = 35\text{-}40$ cells/condition.

Table 1

Potency (IC₅₀, expressed in μ M) and efficacy (defined as maximum drug effect measured at 100 μ M for each drug), n=15-20 cells / condition tested. All measurements are relative to measurements obtained in cells exposed to vehicle (DMSO).

Drug	Wave Suppression		Wave Amplitude		Wave Frequency		Wave Speed	
	Potency (IC ₅₀)	Efficacy (%)	Potency (IC ₅₀)	Efficacy (%)	Potency (IC ₅₀)	Efficacy (%)	Potency (IC ₅₀)	Efficacy (%)
Flecainide	12.8±0.6	70.8	3.6±0.4	50.7	6.5±1	40.3	2.2±0.3	45
Propafenone	20.1±2.4	61	17.4±0.4	28.5	14.3±0.8	33.4	10.9±0.6	24.6
R-Propafenone	2±0.2	70.9	5.5±0.4	40	0.6±0.1	37	7.9±0.6	61.8
S-Propafenone	54.2±18	34.4	24.4±3.5	26.9	21.1±2	12.7	14.6±3	18.7
Encainide	28.7±1.9	40.7	23.3±0.7	28.1	9.0±1.4	28.2	17.3±0.1	24.5
Lidocaine	56.5±2.7	38	26.5±0.2	30.7	26.1±0.01	18	24.2±0.01	32.4
Quinidine	51.7±1.5	36.5	26±0.01	34.2	22.9±0.01	11.3	23±0.002	21.6
Verapamil	37.5±0.1	53.2	45.3±0.3	23	32.5±0.3	30	33.6±0.1	43.3
Procainamide	>100	0	NA	14.1	NA	12.9	NA	18.7
Disopyramide	>100	14.3	NA	21.4	NA	13.4	NA	4.7
Mexiletine	>100	0	NA	33.5	NA	21.6	NA	18
Cibenzoline	>100	18.2	NA	0	NA	36.9	NA	23.6
Ranolazine	>100	6.5	NA	11.9	NA	6.5	NA	22.5
Tetracaine	NA	-29.41	NA	-78.7	28.1±2.6	55.6	31.6±2.2	45.4