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RESEARCH PAPER

Robust anti-arrhythmic efficacy of verapamil and flunarizine against dofetilide-induced TdP arrhythmias is based upon a shared and a different mode of action

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BACKGROUND AND PURPOSE
The high predisposition to Torsade de Pointes (TdP) in dogs with chronic AV-block (CAVB) is well documented. The anti-arrhythmic efficacy and mode of action of Ca^{2+} channel antagonists, flunarizine and verapamil against TdP were investigated.

EXPERIMENTAL APPROACH
Mongrel dogs with CAVB were selected based on the inducibility of TdP with dofetilide. The effects of flunarizine and verapamil were assessed after TdP and in different experiments to prevent dofetilide-induced TdP. Electrocardiogram and ventricular monophasic action potentials were recorded. Electrophysiological parameters and short-term variability of repolarization (STV) were determined. *In vitro*, flunarizine and verapamil were added to determine their effect on (i) dofetilide-induced early after depolarizations (EADs) in canine ventricular myocytes (VM); (ii) diastolic Ca^{2+} sparks in $RyR2^{R4496+/+}$ mouse myocytes; and (iii) peak and late I_{Na} in SCN5A-HEK 293 cells.

KEY RESULTS
Dofetilide increased STV prior to TdP and in VM prior to EADs. Both flunarizine and verapamil completely suppressed TdP and reversed STV to baseline values. Complete prevention of TdP was achieved with both drugs, accompanied by the prevention of an increase in STV. Suppression of EADs was confirmed after flunarizine. Only flunarizine blocked late I_{Na}. Ca^{2+} sparks were reduced with verapamil.

CONCLUSIONS AND IMPLICATIONS
Robust anti-arrhythmic efficacy was seen with both Ca^{2+} channel antagonists. Their divergent electrophysiological actions may be related to different additional effects of the two drugs.

Abbreviations
APD, action potential duration; BVR, beat-to-beat variability of repolarization duration; $[Ca^{2+}]_i$, intracellular calcium concentration; CAVB, chronic AV-block; DADs, delayed afterdepolarizations; EADs, early after depolarizations; $I_{Ca,L}$, L-type calcium current; I_{Kr}, rapid component of the delayed rectifier current; I_{Ks}, slow component

of the delayed rectifier current; late I_{Na}, persistent sodium current; LV, left ventricle; MAP, monophasic action potential; MAPD, duration of the monophasic action potential; MEBs, multiple ectopic beats; NCX, sodium-calcium exchanger; RV, right ventricle; RyR, ryanodine receptor; SEBs, single ectopic beats; SR, sarcoplasmic reticulum; STV_{APD}, short-term variability of repolarization, computed from cellular APD; STV_{LV}, short-term variability of repolarization, computed from LV MAPD; TdP, Torsade de Pointes; VTs, ventricular tachycardias

Introduction

In numerous pro-arrhythmic circumstances, calcium [Ca^{2+}] overload of the sarcoplasmic reticulum (SR) is the cause of Ca^{2+} leak through ryanodine receptors (RyR). The ensuing Ca^{2+} sparks increase cytosolic $[Ca^{2+}]_i$ which in turn may activate the sodium-calcium exchanger (NCX) to generate delayed afterdepolarizations (DADs) and trigger ventricular arrhythmias (de Groot *et al.*, 2000; Pogwizd *et al.*, 2001). The 'Ca^{2+} antagonists', flunarizine and verapamil, suppress DADs or DAD-dependent ventricular tachycardias (VTs) either induced by ouabain (Rosen and Danilo, 1980; Jonkman *et al.*, 1986; Vos *et al.*, 1990; Park *et al.*, 1992) or by cathecholamines (Park *et al.*, 1992). In addition, both drugs have also been shown to be effective against Torsade de Pointes (TdP) arrhythmias, whether seen in congenital (Shimizu *et al.*, 1995) or in acquired long QT syndromes (January *et al.*, 1988; Cosio *et al.*, 1991; Verduyn *et al.*, 1995; Carlsson *et al.*, 1996; Aiba *et al.*, 2005; Gallacher *et al.*, 2007). The latter, however, are more likely initiated by early after depolarizations (EADs). The mechanisms underlying these EADs can be reactivation of $I_{Ca,L}$, a persistent I_{Na} or the NCX-mediated inward current (January *et al.*, 1988; Volders *et al.*, 1997; Sipido *et al.*, 2000; Antoons *et al.*, 2007). Although both flunarizine and verapamil belong to the category of Ca^{2+} antagonists, they have additional actions (Zhang *et al.*, 1999; Trepakova *et al.*, 2006), such as blocking the delayed rectifier current (I_{Kr}) that may negatively affect their anti-arrhythmic efficacy against repolarization-dependent VTs.

The canine model of chronic AV-block (CAVB) has been used to initiate both DAD- and EAD-dependent VTs (Vos *et al.*, 1990; Verduyn *et al.*, 1995; Volders *et al.*, 1997; de Groot *et al.*, 2000; Sipido *et al.*, 2000). The enhanced susceptibility to triggered arrhythmias in this model has been related to SR Ca^{2+} overload and to a diminished repolarization reserve (Vos *et al.*, 1990; de Groot *et al.*, 2000; Sipido *et al.*, 2000; Oros *et al.*, 2008). In this setting, quantification of beat-to-beat variability of repolarization duration (BVR) by short-term variability (STV) has been shown to be a better parameter to predict proarrhythmic predisposition than the QT-time (Thomsen *et al.*, 2006; Oros *et al.*, 2008).

The objectives of this study were to determine if: (i) flunarizine and verapamil prevented and/or suppressed dofetilide-induced TdP in dogs with CAVB; (ii) flunarizine and verapamil improved repolarization reserve, as quantified by STV; (iii) flunarizine was effective against dofetilide-induced increases in STV and EADs in ventricular myocytes isolated from dogs with CAVB; and (iv) their mode of action on Ca^{2+} sparks and late I_{Na} *in vitro* differed.

Methods

General

All animal care and experimental handling was in accordance with the 'European Directive for the Protection of Vertebrate Animals used for Experimental and Scientific Purpose, European Community Directive 86/609/CEE' and under the regulations of The Committee for Experiments on Animals of Utrecht University, the Netherlands.

A total of 26 adult mongrel dogs (Marshall, USA; 23 ± 3 kg, 16 females) were included. Four weeks after induction of complete AV-block, 22 animals were given a dofetilide test (i.v. infusion of 0.025 $mg \cdot kg^{-1}$ for 5 min). In this group, five dogs were excluded because they had TdP at baseline (n = 3) or they were non-inducible (n = 2). Repeatability and reproducibility of arrhythmias have been well studied in this model (Oros *et al.*, 2008). In a second group of four animals with CAVB, verapamil and lidocaine were administered in combination to explore their effect on electrophysiological parameters.

All experiments were performed under complete anesthesia, induced with barbiturates and maintained during the experiments with isoflurane (1.5 %). The detailed description of experimental procedures, AV-node ablation, electrocardiogram and monophasic action potential (MAP) recordings (with MAP duration, MAPD at 90 % repolarization), definitions and data analysis, including determination of STV_{LV} from the left ventricle (LV) MAPD, have been already published (Thomsen *et al.*, 2004; Oros *et al.*, 2006). Early ectopic activity was defined as ectopic beats (EB) initiating before the end of the preceding T wave. Distinction between single (SEBs)

and multiple ectopic beats (MEBs) was made as the latter are considered more proarrhythmic (Belardinelli *et al.*, 2003).

Anti-arrhythmic protocols in vivo

Experimental protocols were performed, separated by at least 2 weeks intervals:

Suppression protocol. Approximately 10 min after the start of dofetilide when TdP was reproducibly seen, flunarizine ($n = 10$; 2 mg·kg^{-1} for 2 min, i.v.) or verapamil ($n = 7$; 0.4 mg·kg^{-1} for 3 min, i.v) were administered to suppress pro-arrhythmic activity.

Validation that TdP remained present in the subsequent 10 min period after dofetilide was recently provided (Oros *et al.*, 2008). To allow comparison of verapamil and flunarizine, the dose of verapamil chosen, had a similar negative inotropic effect in dogs (Vos *et al.*, 1992; Bril *et al.*, 1996) as seen with the anti-arrhythmic dose of flunarizine. Moreover, these equipotent negative haemodynamic effects were confirmed in six sinus rhythm dogs using a 7F catheter (Sentron, Roden, the Netherlands): LV end-systolic pressure decreased by 20% with both drugs: flunarizine from 94 ± 9 to 75 ± 9 mmHg ($P < 0.05$) and verapamil from 87 ± 13 to 67 ± 9 mmHg ($P < 0.05$).

Prevention protocol. Whether vulnerability to dofetilide-induced TdP could be prevented by pre-treatment with flunarizine ($n = 8$) or verapamil ($n = 6$) was investigated in this set of experiments. The dose of dofetilide used was the same as in the previous experiment. Three animals were tested serially with both verapamil and flunarizine.

Combination of drugs. To test if the electrophysiological effects seen with flunarizine were due to a combined block of $I_{Ca,L}$ and late I_{Na}, verapamil (0.2 mg·kg^{-1} for 1.5 min) was followed 5 min later by lidocaine (1.5 mg·kg^{-1} for 1 min), a preferential late I_{Na} blocker (Fredj *et al.*, 2006).

In vitro *experiments*

The following concentrations of drugs were used: 1 μM dofetilide, 1 μM and 10 μM flunarizine or verapamil.

Effects of flunarizine on cellular STV. Single myocytes from CAVB dogs were enzymically isolated (Volders *et al.*, 1998). Action potentials were triggered in whole-cell current clamp mode with 2 ms current injections at a cycle length of 2000 ms and recorded with PClamp9 software (Molecular Devices, Sunnyvale, CA, USA). Action potential duration (APD) was measured at 90% repolarization and cellular STV was calculated from 20 successive APDs (STV_{APD}) similar to the *in vivo* quantification-(Volders *et al.*, 1998; Thomsen *et al.*, 2004). Experiments were performed in Tyrode solution containing (in mmol·L^{-1}): 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 11.8 HEPES and 10 glucose, pH 7.4. Pipettes had a resistance of 2–3 MΩ when filled with pipette solution, containing (in mmol·L^{-1}): 130 KCl, 10 NaCl, 10 HEPES, 5 MgATP and 0.5 $MgCl_2$, pH 7.2. As with the *in vivo* experiments, two experimental protocols were used:

Protocol 1: effects of flunarizine on baseline cellular APD and STV in eight myocytes isolated from the LV of four dogs.

Protocol 2: effects of flunarizine on dofetilide-induced EADs. If 1 μM dofetilide induced EADs, flunarizine was added to the Tyrode solution to test its suppressive effect on EADs and dofetilide-increased APD and STV. For these experiments another eight cells [$n = 4$ right ventricle (RV) and $n = 4$ LV] from five dogs were used.

Effects of flunarizine and verapamil on I_{Na}. For recording cardiac peak and late I_{Na}, SCN5A-HEK 293 cells (expressing $Na_v1.5$) were superfused with bath solution containing (in mmol·L^{-1}): 140 NaCl, 4.0 KCl, 1.8 $CaCl_2$, 0.75 $MgCl_2$ and 5 HEPES (pH adjusted to 7.4 with NaOH). The pipette solution contained (in mmol·L^{-1}): 20 CsCl, 120 CsF, 2 EGTA and 5 HEPES (pH adjusted to 7.4 with CsOH). All experiments were performed at 21 ± 1°C. Whole-cell membrane current was recorded as previously described (Hamill *et al.*, 1981). Computer software (pCLAMP 10.0, Molecular Devices, Sunnyvale, CA) was used to generate voltage-clamp protocols. Patch-clamp amplifier (Multiclamp 700B, Molecular Devices) data were sampled at 5 kHz. Whole-cell capacitance was compensated using the internal voltage-clamp circuitry and about 75–80% of series resistance was compensated. Membrane potentials were not corrected for junction potentials that arise between the pipette and bath solution. Cells were held at –140 mV and dialysed for 5 min before I_{Na} recording. Data analysis of all measured currents was performed using pCLAMP 10.0 and Origin 7.0 (MicroCal, Northampton, MA) software. To measure the extent of tonic block (first-pulse) by flunarizine or verapamil on peak I_{Na}, 24-ms depolarizing steps to –20 mV from a holding potential of –140 mV were applied to cells at a rate of 0.1 Hz. The magnitude of peak I_{Na} in the presence of drug was normalized to the respective control value. To measure the effect of flunarizine or verapamil on late I_{Na}, the normally small late I_{Na} was augmented by exposure of cells to Anemone Toxin-II (ATX-II; 3 nM; Song *et al.*, 2008), and the effect of drug to reduce the ATX-II-induced

late I_{Na} was determined. Late I_{Na} was defined as the magnitude of I_{Na} between 200 and 220 ms after application of a 220-ms depolarizing step to –20 mV from a holding potential of –140 mV applied at a rate of 0.1 Hz.

Effects of flunarizine and verapamil on Ca^{2+} sparks. Abnormally high spontaneous Ca^{2+} release in diastole (Ca^{2+} sparks) were recorded in intact quiescent myocytes enzymically isolated from homozygous mice carrying the mutation R4496C of the cardiac ryanodine receptor ($RyR2^{R4496C+/+}$), which underlies catecholamine polymorphic ventricular tachycardia (CPVT) (Priori *et al.*, 2001; Fernandez-Velasco *et al.*, 2009). To measure the effects of verapamil and flunarizine on spontaneous Ca^{2+} spark activity, cells were loaded with fluorescent Ca^{2+} indicator (Fluo-3 AM) as previously described (Fernandez-Velasco *et al.*, 2009). Cells were recorded under continuous perfusion with Tyrode solution (in mmol·L^{-1}: 140 NaCl, 4 KCl, 1.1 $MgCl_2$, 10 HEPES, 10 glucose, 1.8 $CaCl_2$; pH = 7.4, with NaOH) before and following the addition of flunarizine or verapamil for 10 min.

Images were obtained by confocal microscopy (Meta Zeiss LSM 510, objective w.i. 63x, n.a. 1.2) in the line scan mode as previously explained. Image analyses were performed by homemade routines using IDL software (Research System Inc.). Images were corrected for the background fluorescence.

Statistical analysis

Pooled data are expressed as mean ± standard deviation except the result on I_{Na} where results are presented as mean ± SEM. For the effects of drugs over time, comparisons were performed using a one-way repeated-measures ANOVA followed by a Bonferroni correction. For non-parametric comparisons, the Kruskal–Wallis test was used.

Materials

Flunarizine was supplied by Janssen Pharmaceutica N.V.; verapamil (Isoptin) and isoflurane was from Abbott, Europe; lidocaine from Braun Melsungen AG, Germany. ATX-II and all other chemicals were from Sigma. Channel, receptor and drug nomenclature follows Alexander *et al.* (2009).

Results

Antiarrhythmic effects of flunarizine

Flunarizine suppression. Dofetilide induced TdP with a median duration of 6.8 s, after 3.1 ± 1 min. After adding flunarizine, all arrhythmias disappeared with the exception of some SEBs in one dog (Figure 1 and Table 1). These anti-arrhythmic effects remained present for more than 10 min. Thereafter, some MEBs returned, albeit less severe. Electrophysiologically, dofetilide increased all repolarization parameters (QT, QT_C, LVMAPD and RVMAPD) and STV_{LV} before the first EB (2.5 ± 0.5 min. after start dofetilide). Flunarizine decreased the dofetilide-augmented STV_{LV} and all the other repolarization parameters, to a level similar to baseline (Table 1 upper part and Figure 1).

Flunarizine prevention. Pretreating the same animals with flunarizine resulted in complete prevention of TdP (Figure 2, upper part). During a 10 min. period, dofetilide could only induce few single EBs (6 ± 10 beats/10 min). Flunarizine significantly decreased baseline STV_{LV} and QTc. After adding dofetilide, STV_{LV} remained at a level similar to baseline, whereas an increase in QTc could not be prevented by this drug completely (Figure 2 and Table 1, lower part).

Effect of flunarizine on baseline cellular BVR and on dofetilide-induced EADs. In untreated isolated myocytes from dogs with CAVB, flunarizine shortened (at 10 min) both APD (from 418 ± 116 ms in baseline to 312 ± 74 ms, $P < 0.05$) and cellular STV_{APD} (baseline 20 ± 10 ms to 11 ± 4 ms, $P < 0.05$). The time course of changes in APD and STV during an experiment is shown in Figure 3A.

In dofetilide-treated cells, APD increased from 337 ± 119 to 507 ± 153 ms ($P < 0.05$) and STV from 14 ± 14 to 65 ± 34 ms ($P < 0.05$). EADs occurred in eight from a total of nine cells. Addition of flunarizine suppressed all dofetilide-induced EADs (from 8/8 to 0/8, $P < 0.05$) and reversed APD (289 ± 60 ms) and cellular STV (11 ± 5 ms) to baseline values ($P > 0.5$ vs. baseline). A representative example is shown in Figure 3B.

Antiarrhythmic effects of verapamil

Verapamil suppression. Similar arrhythmia was seen with dofetilide in this group: TdP induction after 3.7 ± 1 min with a median duration of 7.1 s. All TdP and MEBs were suppressed, while some SEBs remained in three dogs (Table 2, upper part). Verapamil did not affect the dofetilide-prolonged repolarization duration (QT, QTc, LVMAPD and RVMAPD) but reduced the variability of repolarization STV_{LV} to a level similar to control (Table 2, upper part).

Verapamil prevention. Verapamil pretreatment also prevented TdP induction remarkably, only one self-terminating TdP was seen. However, dofetilide was

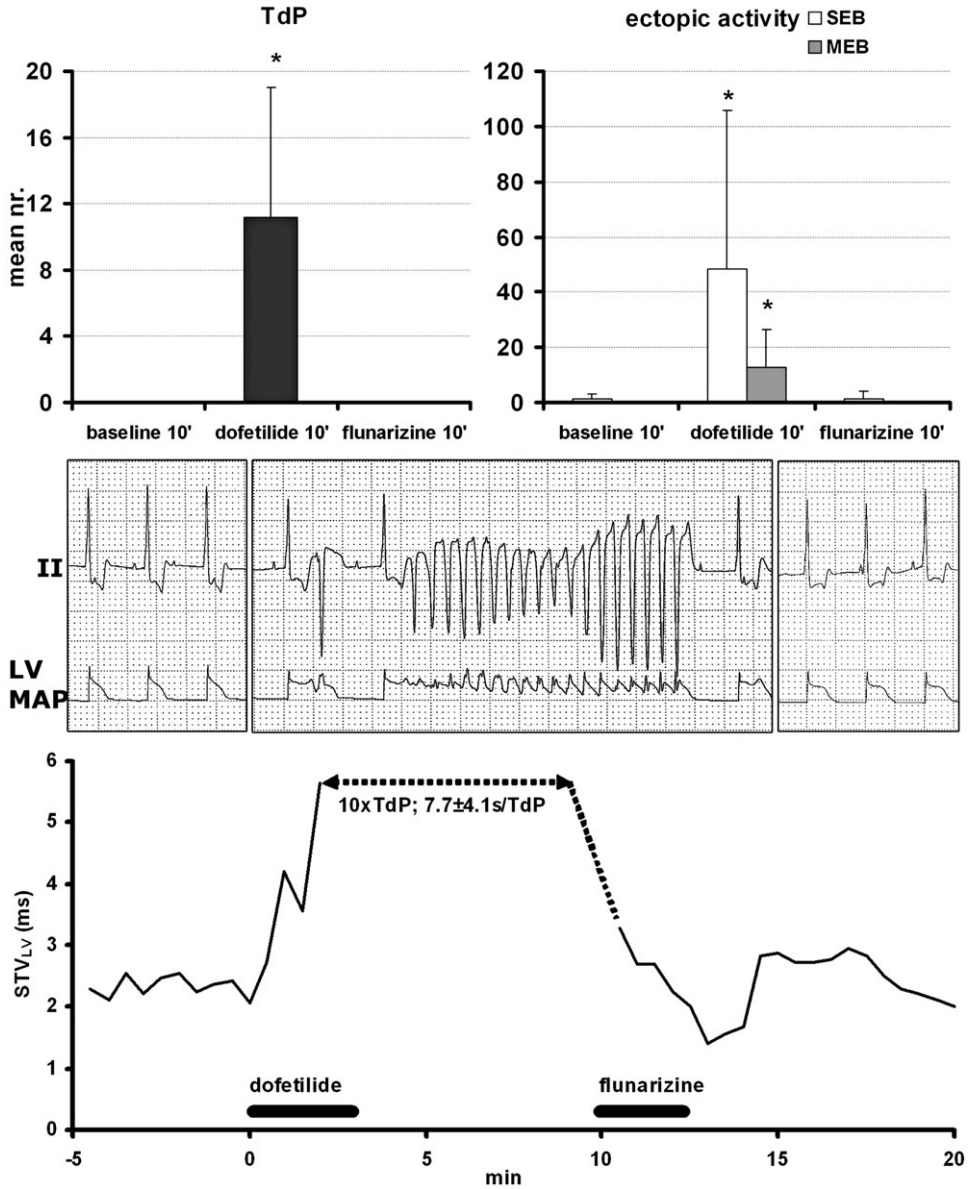

Figure 1

Upper panel: anti-arrhythmic effects of flunarizine (suppression) against dofetilide-induced TdP (left) and ectopic activity (right; as single ectopic beats, SEB and multiple ectopic beats, MEB) is shown with an individual example (middle part) of lead II electrocardiogram (ECG) and left ventricular monophasic action potential (LV MAP) recordings (printed at 10 mm·s^{-1} speed and calibrated at 1 mV per cm for ECG and 20 mV for the MAP recording) on scale paper in baseline (left), with TdP (middle) and after flunarizine. Lower panel illustrates continuous short-term variability (STV$_{LV}$) quantification for this experiment. *P < 0.05 versus baseline. TdP, Torsade de Pointes.

still able to generate numerous SEBs and few MEBs (Table 2, lower part). Verapamil did not change baseline electrophysiological parameters, or STV$_{LV}$. The duration of repolarization parameters (QT, QTc, LVMAPD and RVMAPD) was prolonged after adding dofetilide despite verapamil pretreatment. However, the variability of repolarization (STV$_{LV}$) was not significantly increased after dofetilide (Table 2, lower part).

Analysis of the mode of action

Effects of flunarizine and verapamil on I_{Na}. Figure 4 shows the effect of flunarizine (Figure 4A, left) and verapamil (Figure 4B, right) on late I_{Na} induced by ATX-II (Figure 4). Flunarizine (1 μM, Figure 4A) inhibited late I_{Na} by 94.4 ± 2.3%, n = 5 cells, P < 0.05). However, at a higher concentration (10 μM), flunarizine had no effect on peak I_{Na} (tonic block; 4.3 ± 3.0%, n = 4 cells, P > 0.05). In contrast to

Table 1

Summary of electrophysiological parameters and arrhythmic events in flunarizine suppression and prevention experiments

	Baseline 1	Dofetilide	Flunarizine
RR	1181 ± 87	1291 ± 140	1219 ± 251
QT	436 ± 44	566 ± 29*	435 ± 36$
QT_C	421 ± 49	553 ± 40*	425 ± 38$
LV MAPD	355 ± 35	492 ± 53*	367 ± 42$
RV MAPD	310 ± 32	395 ± 68*	333 ± 30$
ΔMAPD	51 ± 28	97 ± 56*	48 ± 32$
STV_{LV}	1.8 ± 0.5	4.5 ± 1.5*	1.5 ± 0.6$
TdP	0 ± 0	11 ± 8*	0 ± 0$
MEB	0 ± 0	13 ± 14*	0 ± 0$
SEB	1 ± 2	48 ± 58*	1 ± 3$
	Baseline 2	**Flunarizine**	**Dofetilide**
RR	1239 ± 329	1291 ± 390	1410 ± 462
QT	422 ± 51	380 ± 50*	494 ± 92*#
QT_C	413 ± 51	369 ± 41*	476 ± 77*#
LV MAPD	299 ± 44	277 ± 36	380 ± 65*#
RV MAPD	286 ± 39	275 ± 44	348 ± 73*#
ΔMAPD	42 ± 27	22 ± 16	38 ± 42
STV_{LV}	1.5 ± 0.6	1.0 ± 0.5*	1.4 ± 0.5
TdP	0 ± 0	0 ± 0	0 ± 0
MEB	0 ± 1	0 ± 0	0 ± 0
SEB	3 ± 6	3 ± 5	6 ± 10

Maximal effects of flunarizine (5 min) in suppression experiments (upper part) and at the end of the infusion (2 min) in prevention experiments are shown (lower part). Arrhythmias are quantified as average number of events (TdP, MEB, SEB) per 10 min, except for the pretreatment with flunarizine (lower part) where after 5 min dofetilide was added. All electrophysiological parameters are expressed in ms and arrhythmias as average number per time interval.

*$P < 0.05$ versus baseline; $^{\$}P < 0.05$ versus dofetilide; $^{\#}P < 0.05$ versus flunarizine pretreatment.

LV, left ventricle; MAPD, duration of the monophasic action potential; MEB, multiple ectopic beat; RV, right ventricle; SEB, single ectopic beat; STV_{LV}, short term variability of repolarization, computed from LV MAPD; TdP, Torsade de Pointes.

flunarizine, verapamil (Figure 4B, 10 μM) had no effect on either late I_{Na} ($n = 5$ cells, $P > 0.05$) or peak I_{Na} (tonic block; 10 μM, $n = 6$ cells ($P > 0.05$ and 30 μM, $n = 4$ cells, $P > 0.05$), as compared with control.

Ca^{2+} sparks study. Acute application of these drugs on the frequency of spontaneous Ca^{2+} sparks in cardiac myocytes expressing a gain-of-function mutation in the RyR2 was examined. Flunarizine (1 μM) did not change the frequency of spontaneous Ca^{2+} sparks in RyR^{R4496C} cells (Figure 5A, $P > 0.05$). In contrast, verapamil (10 μM) significantly reduced spontaneous Ca^{2+} spark activity by ≈35% (Figure 5B, $P < 0.005$).

In vivo *effects of verapamil in combination with lidocaine.* To confirm that STV_{LV} reduction in baseline by flunarizine was in part due to inhibition of late I_{Na}, the effects of verapamil combined with lidocaine were explored. The combination of these drugs shortened the duration of repolarization (QTc from 353 ± 35 to 306 ± 21 ms, $P < 0.05$) and STV_{LV} was significantly reduced, effects similar to those seen with flunarizine alone (Figure 6).

Discussion

The most important findings of this study can be summarized as follows: (i) both Ca^{2+} antagonists flunarizine and verapamil were equally and markedly effective in suppressing and preventing dofetilide-induced TdP; (ii) this anti-arrhythmic effect was reflected in STV_{LV}, but not in QT or LV MAPD; (iii) flunarizine but not verapamil decreased

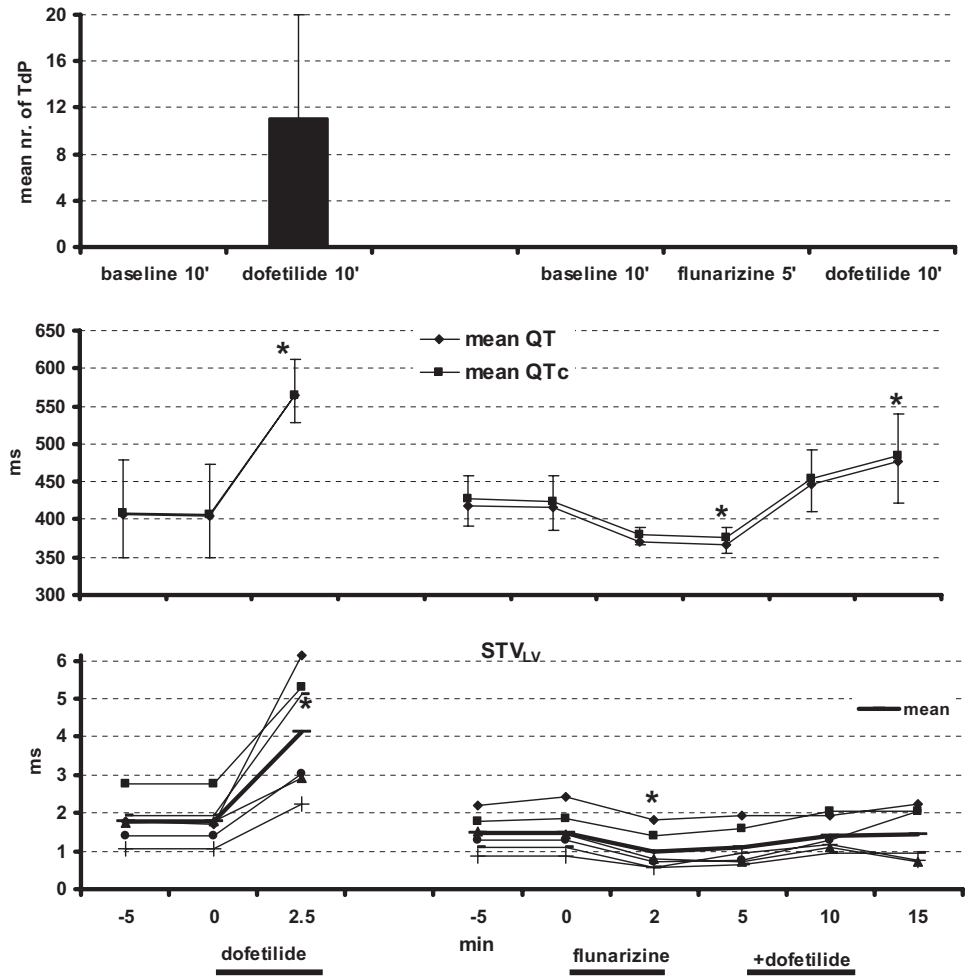

Figure 2

TdP prevention (upper panel) with flunarizine is presented in two serial experiments, first dofetilide alone (left) and with flunarizine pretreatment (right). The effects on QT/QTc (middle part) and short-term variability (STV_{LV}) in individuals as well as average (lower part) are plotted. TdP, Torsade de Pointes.

BVR in baseline, which could be ascribed to its additional late I_{Na} blocking effect; and (iv) verapamil modestly reduced Ca^{2+} sparks, an effect not seen with flunarizine.

Repolarization-dependent ventricular arrhythmias and EADs

The common Ca^{2+} antagonism of verapamil and flunarizine was used to investigate whether they could improve repolarization reserve, as reflected in protection against dofetilide-induced TdP. These repolarization-dependent arrhythmias normally occur under conditions in which this reserve is 'challenged beyond capacity', as in the long QT syndromes, or in congestive heart failure (Tomaselli *et al.*, 1994). Lately, it has been suggested that this reserve can be estimated by STV (Thomsen *et al.*, 2004; 2006; 2007; Hinterseer *et al.*, 2008; Oros *et al.*, 2008). EADs and EAD-dependent triggered activity have been considered as possible initiation mechanisms for TdP in long QT syndromes (el-Sherif *et al.*, 1996; Belardinelli *et al.*, 2003). To develop new antiarrhythmic drugs, it is important to understand possible targets that are key components of the generation of EADs, such as L-type Ca^{2+} channels, Na^+ channels (with peak and late I_{Na}), RyR and its regulating unit calstabin2 (FKBP12.6), and the NCX. EADs have at least two different ways in which they may be generated: (i) window currents, either through the $I_{Ca,L}$ or late I_{Na}; and (ii) NCX-mediated inward currents due to an abnormal Ca^{2+} release from the SR. The $I_{Ca,L}$ has been studied extensively as block of $I_{Ca,L}$ by verapamil or nitrendipine prevented EADs from developing (Marban *et al.*, 1986; January *et al.*, 1988) and as regional differences in the expression levels of L-type Ca^{2+} channels have implications for the origin of EADs (Sims *et al.*, 2008). This effect may also explain why I_{Kr} blockade by

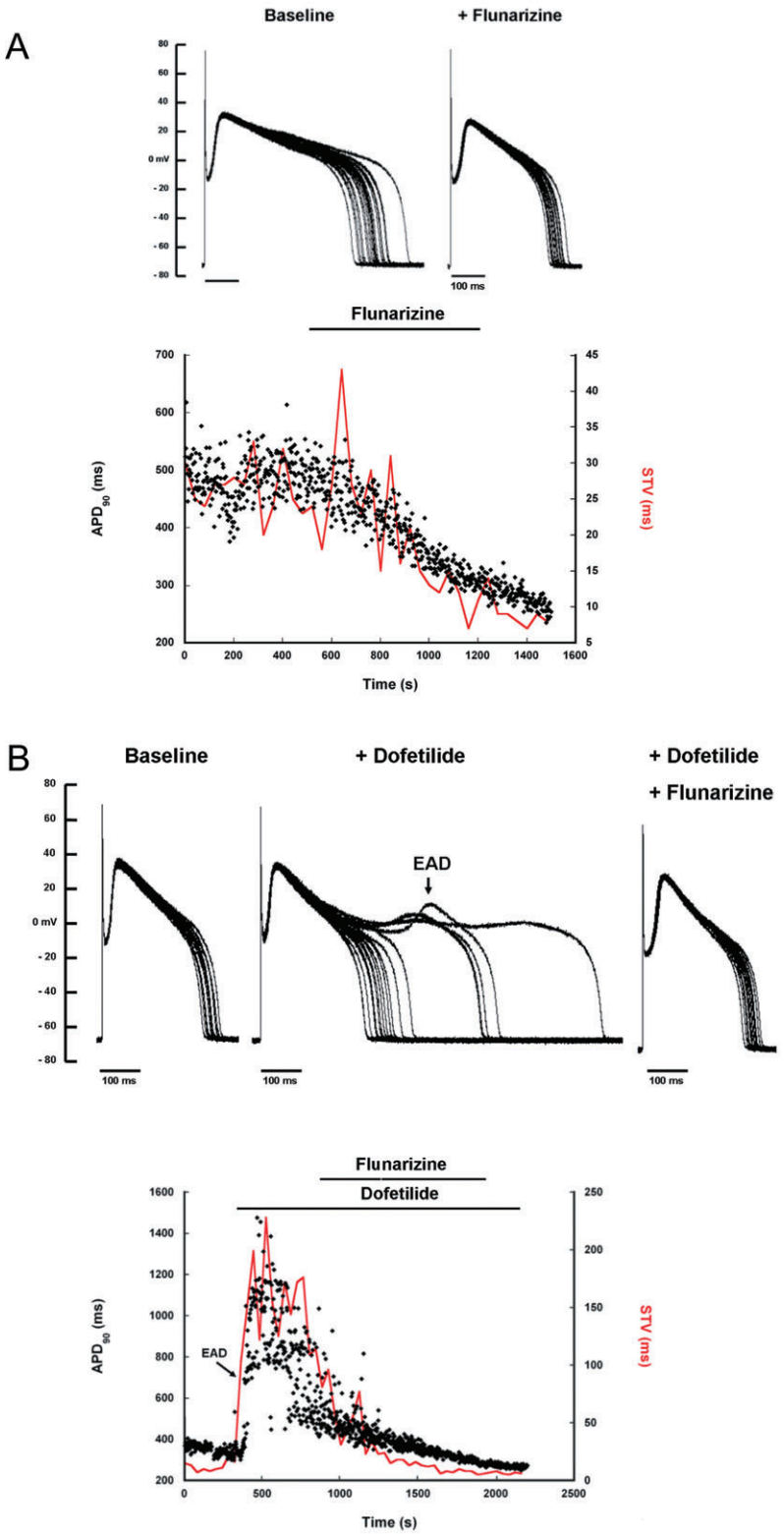

Figure 3

Anti-arrhythmic effects of flunarizine in isolated ventricular myocytes of the chronic AV-block (CAVB) dog are depicted. (A) 20 superimposed consecutive action potentials (APs) in baseline (left) and after flunarizine (right) as well as the time course of APD (dots) and short-term variability (STV_{APD}, continuous red line), baseline and with flunarizine perfusion are shown. (B) Similar, 20 superimposed APs in baseline (left), with dofetilide-induced EADs (arrow in middle panel) and after EADs suppression with flunarizine (right) and the temporal behaviour of APD and STV_{APD} are shown for this experiment. EADs, early after depolarizations.

Table 2

Summary of electrophysiological parameters and arrhythmic events in verapamil suppression and prevention experiments

	Baseline 1	Dofetilide	Verapamil
RR	1361 ± 190	1520 ± 210*	1476 ± 166
QT	456 ± 67	611 ± 92*	557 ± 97*
QT_C	424 ± 62	566 ± 87*	516 ± 90*
LV MAPD	349 ± 88	505 ± 110*	466 ± 95*
RV MAPD	305 ± 63	446 ± 135*	392 ± 108*
ΔMAPD	44 ± 39	72 ± 36	92 ± 92
STV_{LV}	1.7 ± 0.4	3.2 ± 1.1*	1.5 ± 0.7$^{\$}$
TdP	0 ± 0	9 ± 5*	0 ± 0
MEB	0 ± 0	9 ± 4*	0 ± 0
SEB	1 ± 1	50 ± 31*	9 ± 15
	Baseline 2	**Verapamil**	**Dofetilide**
RR	1285 ± 202	1212 ± 228	1464 ± 240$^{\#}$
QT	442 ± 71	436 ± 57	651 ± 47*$^{\#}$
QT_C	417 ± 58	417 ± 41	611 ± 34*$^{\#}$
LV MAPD	332 ± 68	328 ± 34	554 ± 77*$^{\#}$
RV MAPD	324 ± 43	318 ± 37	545 ± 53*$^{\#}$
ΔMAPD	32 ± 29	33 ± 16	30 ± 16
STV_{LV}	1.3 ± 0.4	1.4 ± 0.6	2.3 ± 1.4
TdP	0 ± 0	0 ± 0	0.2 ± 0.4
MEB	0 ± 1	0.2 ± 0.4	3 ± 6
SEB	2 ± 4	1 ± 2	28 ± 44

Maximal effects of verapamil in suppression experiments (at 10 min, upper part) and at the end of the infusion (3 min) in prevention experiments are shown (lower part). All electrophysiological parameters are expressed in ms and arrhythmias (TdP, MEB, SEB) as average number per time interval.
*$P < 0.05$ versus baseline; $^{\$}P < 0.05$ versus dofetilide; $^{\#}P < 0.05$ versus verapamil pretreatment.
LV, left ventricle; MAPD, duration of the monophasic action potential; MEB, multiple ectopic beat; RV, right ventricle; SEB, single ectopic beat; STV_{LV}, short term variability of repolarization, computed from LV MAPD; TdP, Torsade de Pointes.

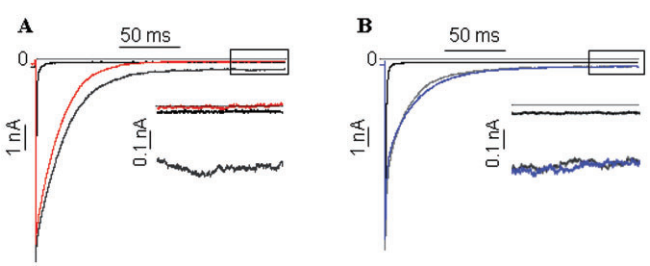

Figure 4

Effects of flunarizine (left) and verapamil (right) on late I_{Na}: representative recordings of late I_{Na} from a single cell in the absence of drug (black line), during superfusion with 3 nM ATX-II (ATX, grey line) and during superfusion with 1 μM flunarizine (left; red line) or 10 μM verapamil (right; blue line). Insets: expanded traces (last 50 ms following depolarizing pulse) of late I_{Na} in the absence (black line) and presence of flunarizine (red line) or verapamil (blue line) respectively.

verapamil (Zhang *et al.*, 1999) and flunarizine are not pro-arrhythmic because an additional block of $I_{Ca,L}$ protects the heart from developing EADs, despite the QT lengthening induced by I_{Kr} block (Bril *et al.*, 1996). This balance between $I_{Ca,L}$ recovery and ventricular repolarization serves also as a physiological stabilizer (Guo *et al.*, 2008).

The second theory, the involvement of abnormal SR Ca^{2+} release in generating EADs, is more controversial (Volders *et al.*, 1997; Antoons *et al.*, 2007). Nevertheless there is evidence that DADs and EADs may occur in the same preparation (Marban *et al.*, 1986; Priori and Corr, 1990; Volders *et al.*, 1997; Song *et al.*, 2008), suggesting a similar etiology. Moreover, EADs and calcium transients have been related (Hamlin and Kijtawornrat, 2008). Indirectly, activation of calcium/calmodulin-dependent protein kinase II (CaMKII) due to an increase in

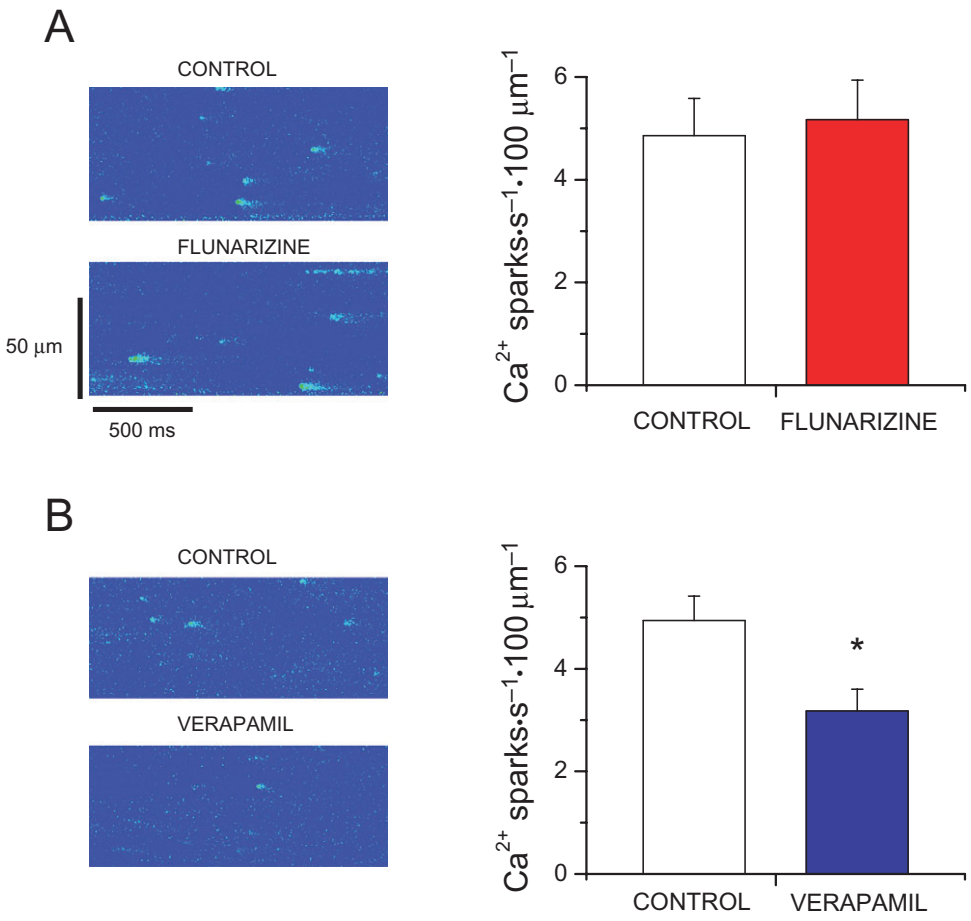

Figure 5

(A) Left, representative line-scan images of spontaneous Ca^{2+} sparks recorded in a $RyR2^{R4496C+/+}$ ventricular myocyte in the absence (top) or presence (bottom) of 1 µM flunarizine. Right, Ca^{2+} spark occurrence before (control) and during flunarizine (n = 8 cells). (B) Similar, images of spontaneous Ca^{2+} sparks in the absence (top) or presence (bottom) of 10 µM verapamil. Right panel shows the average data in control and with verapamil (n = 11 cells). *P < 0.05 versus control. RyR, ryanodine receptor.

$[Ca^{2+}]_i$ might facilitate both $I_{Ca,L}$ and I_{Na}, thus inducing EADs and DADs (Anderson *et al.*, 1998). Because of the number of actions of the drugs, the relevance of this alternative (for the conditional phase) was difficult to assess. However, measuring diastolic Ca^{2+} sparks known to underlie DAD generation is an interesting approach to address the question of whether flunarizine and verapamil inhibit the disturbed SR Ca^{2+} release events.

Calcium antagonists: flunarizine and verapamil

Flunarizine does not belong to the cardiovascular categories of Ca^{2+} antagonists. Clinically, the drug has been used to treat neurological disorders, such as migraine and has been termed a calcium overload blocker (van Zwieten, 1986). The latter implies that flunarizine may have an intracellular target. However, until now, only sarcolemmal effects have been described. Besides blocking three types of Ca^{2+} channels (Tytgat *et al.*, 1991; 1996), $I_{Ca,L}$ (IC_{50} = 4.6–10 µM), $I_{Ca,N}$ (0.8 µM) and $I_{Ca,T}$ (3.3–11 µM), flunarizine is also a potent I_{Kr} (5.7 nM) and I_{Ks} (0.7 µM) blocker (Trepakova *et al.*, 2006).

Verapamil is known to block $I_{Ca,L}$ (0.6–15.5 µM) (Hosey and Lazdunski, 1988), I_{Kr} (0.1 µM) (Zhang *et al.*, 1999) and I_{Ks} (5.7–6.3 µM) (Aiba *et al.*, 2005). According to the supplier, our dose of flunarizine will reach a total plasma concentration around 1.7 µM (828 ng·mL^{-1}, MW 477.4) while, for verapamil, this value is around 0.5 µM (Fossa *et al.*, 2002).

In susceptible dogs with CAVB, both drugs were very effective (100% efficacy) in preventing and terminating dofetilide-induced TdP. They were much more effective stronger than other drugs such as the late I_{Na} blockers, ranolazine and lidocaine, which were effective in approximately 60% of the animals (Antoons *et al.*, 2010), whereas the $I_{K,ATP}$ agonist levcromakalim was slightly more effective (70%,

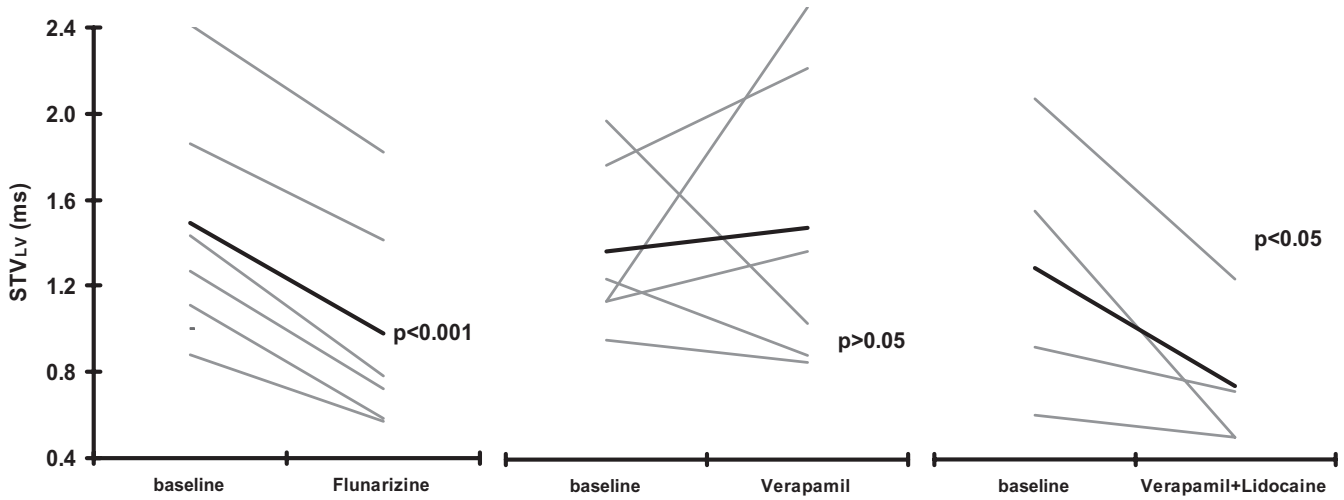

Figure 6

Effects of flunarizine (left), verapamil (middle) and the combination of verapamil and lidocaine on baseline short-term variability (STV_{LV}). Effects of these drugs on baseline STV_{LV} is shown for individual animals (thin lines) and as a mean value for each group (thick line).

unpublished data). This confirms that inhibition of $I_{Ca,L}$ is a very effective way to treat dofetilide-induced TdP, assuming that no other actions are involved (see below).

Additional modes of action

In order to gain more insight in the mode of action of flunarizine and verapamil, we undertook cellular experiments to determine their possible action against late I_{Na} and Ca^{2+} sparks. Flunarizine but not verapamil blocked late I_{Na}, which could explain why flunarizine, but not verapamil, was effective against veratridine-induced contractures (Patmore *et al.*, 1989).

Albeit modestly, verapamil (at high concentrations) but not flunarizine could reduce the frequency of diastolic Ca^{2+} sparks in a cell model expressing a gain-of-function mutation in the RyR2 responsible for abnormal Ca^{2+} leakage. These results show that the antiarrhythmic molecular mechanism of verapamil and flunarizine could involve different targets. Regarding flunarizine it is possible to discard an action of flunarizine on RyR2 activity. As mentioned, there is controversy concerning the proposition that SR Ca^{2+} leak may indirectly provide inward currents that contribute to induction of EADs (Fauconnier *et al.*, 2005; Hamlin and Kijtawornrat, 2008). One way to study this is by application of drugs that specifically block this release, like ryanodine or K201. Unfortunately, both drugs do not have a high degree of specificity.

When evaluating the literature concerning ryanodine and its action on DADs or EADs, it becomes apparent that the results are not consistent. Ryanodine is known to be anti-arrhythmic against DADs and DAD-dependent VT (Marban *et al.*, 1986; Priori and Corr, 1990). In dogs with CAVB, ryanodine (10 mg) was effective against drug-induced TdP, whereas ryanodine was not effective against cesium or ATX-II-induced EADs (Marban *et al.*, 1986; Park *et al.*, 1992; Song *et al.*, 2008), but anti-arrhythmic against catecholamine-induced EADs (Priori and Corr, 1990). Ryanodine and flunarizine were both effective against acceleration-induced EADs (Burashnikov and Antzelevitch, 1998). Until there is a specific blocker for unconditional Ca^{2+} leak, it will be difficult to prove SR leakage to be part of the generation of EADs. It is evident however, that adding blocking properties against either late I_{Na} or Ca^{2+} sparks could generate more anti-arrhythmic 'strength'. Future studies are necessary to evaluate which of the two additional actions is the most attractive.

Anti-arrhythmic action and STV. The anti-arrhythmic potential of flunarizine and verapamil was clearly reflected by the changes in STV_{LV}. Its suppressive actions were associated with a reduction in STV_{LV}, whereas the preventive effects could be seen in keeping STV_{LV} at low(er) levels. Anti-arrhythmic properties of flunarizine were confirmed *in vitro* on drug-induced EADs and STV_{APD}. Thus, STV is an indicator of the ability of the heart to withstand a pro-arrhythmic challenge. However, the action on the other electrophysiological parameters differed. Flunarizine showed a pronounced action on repolariza-

tion parameters such as QTc and LV MAPD and cellular APD, whereas the effect of verapamil on repolarization time was much smaller or even absent.

Second, flunarizine decreased baseline STV_{LV}, suggesting that this drug may increase repolarization reserve. This interpretation is consistent with the greater effect of verapamil combined with lidocaine on STV_{LV} (Figure 6) and QT LV MAPD than verapamil alone.

The fact that flunarizine decreased APD/QTc while verapamil did not, could in part contribute to the mechanism by which flunarizine reduced STV_{LV} or STV_{APD}. However, the contribution of APD to STV_{LV} was not seen in the suppression experiments where verapamil shortened STV without a significant reduction in APD or QTc.

Study limitations

The results obtained in the SCN5A-HEK 293 cells and/or in myocytes isolated from the transgenic mouse model $RyR2^{R4496C+/+}$ may differ from the results that could be obtained from myocytes isolated from CAVB dog hearts.

The blocking effect of verapamil on Ca^{2+} sparks was not very robust and attained at relatively high dosages. Whether this finding has therapeutic consequences is therefore unclear.

In conclusion, a robust anti-arrhythmic efficacy was seen with flunarizine and verapamil. This suppressive and preventive action of the drugs was reflected in STV_{LV} or cellular STV_{APD}. Their different electrophysiological response may be related to different additional effects of the two drugs: flunarizine blocks late I_{Na}, whereas verapamil reduces Ca^{2+} sparks.

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Within the Contica framework we received the knock-in mice $RyR2^{4496C+/+}$ from Drs S. G. Priori and C. Napolitano, Pavia, Italy.

Conflicts of interest

SR and LB are employees of Gilead Sciences Inc.

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