ICaL inhibition prevents arrhythmogenic Ca\textsuperscript{2+} waves caused by abnormal Ca\textsuperscript{2+} sensitivity of RyR or SR Ca\textsuperscript{2+} accumulation

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\textbf{Aims} Arrhythmogenic Ca\textsuperscript{2+} waves result from uncontrolled Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) that occurs with increased Ca\textsuperscript{2+} sensitivity of the ryanodine receptor (RyR) or excessive Ca\textsuperscript{2+} accumulation during β-adrenergic stimulation. We hypothesized that inhibition of the L-type Ca\textsuperscript{2+} current (ICaL) could prevent such Ca\textsuperscript{2+} waves in both situations.

\textbf{Methods and results} Ca\textsuperscript{2+} waves were induced in mouse left ventricular cardiomyocytes by isoproterenol combined with caffeine to increase RyR Ca\textsuperscript{2+} sensitivity. ICaL inhibition by verapamil (0.5 μM) reduced Ca\textsuperscript{2+} wave probability in cardiomyocytes during electrostimulation, and during a 10 s rest period after ceasing stimulation. A separate type of Ca\textsuperscript{2+} release events occurred during the decay phase of the Ca\textsuperscript{2+} transient and was not prevented by verapamil. Verapamil decreased Ca\textsuperscript{2+} spark frequency, but not in permeabilized cells, indicating that this was not due to direct effects on RyR. The antiarrhythmic effect of verapamil was due to reduced SR Ca\textsuperscript{2+} content following ICaL inhibition. Computational modelling supported that the level of ICaL inhibition obtained experimentally was sufficient to reduce the SR Ca\textsuperscript{2+} content. Ca\textsuperscript{2+} wave prevention through reduced SR Ca\textsuperscript{2+} content was also effective in heterozygous ankyrin B knockout mice with excessive SR Ca\textsuperscript{2+} accumulation during β-adrenergic stimulation.

\textbf{Conclusion} ICaL inhibition prevents diastolic Ca\textsuperscript{2+} waves caused by increased Ca\textsuperscript{2+} sensitivity of RyR or excessive SR Ca\textsuperscript{2+} accumulation during β-adrenergic stimulation. In contrast, unstimulated early Ca\textsuperscript{2+} release during the decay of the Ca\textsuperscript{2+} transient is not prevented, and merits further study to understand the full antiarrhythmic potential of ICaL inhibition.

\textbf{Keywords} L-type Ca\textsuperscript{2+} current • Ca\textsuperscript{2+} homeostasis • sarcoplasmic reticulum function • Ca\textsuperscript{2+} waves • Ca\textsuperscript{2+} sparks

1. Introduction

Triggered arrhythmias can result from delayed afterdepolarizations (DADs), i.e. depolarizations of the cell membrane in phase 4 of the action potential.\textsuperscript{1} DADs occur when the Na\textsuperscript{+},Ca\textsuperscript{2+}-exchanger (NCX) is activated by Ca\textsuperscript{2+} released from the sarcoplasmic reticulum (SR) in diastole. The magnitude of DADs depends on the amount of Ca\textsuperscript{2+} released from the SR.\textsuperscript{2} For the release to trigger a spontaneous action potential, local release, i.e. a Ca\textsuperscript{2+} spark, must initiate a self-propagating process of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, called a Ca\textsuperscript{2+} wave. Ca\textsuperscript{2+} waves can occur only when the SR Ca\textsuperscript{2+} content ([Ca\textsuperscript{2+}]\textsubscript{SR}) reaches a threshold level set by the SR Ca\textsuperscript{2+} release channel, the ryanodine receptor (RyR). [Ca\textsuperscript{2+}]\textsubscript{SR} that exceeds this threshold, i.e. ‘Ca\textsuperscript{2+} overload’, is a prerequisite for DADs.\textsuperscript{3} However, suprathreshold [Ca\textsuperscript{2+}]\textsubscript{SR} can result from different perturbations of Ca\textsuperscript{2+} homeostasis: lowered threshold for diastolic SR Ca\textsuperscript{2+} release due to increased Ca\textsuperscript{2+} sensitivity of RyR, or increased loading of the SR due to excessive Ca\textsuperscript{2+} accumulation or decreased Ca\textsuperscript{2+} efflux from the cell.\textsuperscript{4} We hypothesized that reduced influx of Ca\textsuperscript{2+} through the L-type Ca\textsuperscript{2+} channel (LTCC) could prevent
Ca\(^{2+}\) overload in both conditions, making the L-type Ca\(^{2+}\) current (\(I_{\text{calc}}\)) an appropriate therapeutic target.

New therapeutic strategies for triggered arrhythmias are needed since the current treatment with \(\beta\)-blockers is insufficient in some patients.\(^5\) \(I_{\text{calc}}\) inhibition has been proposed as an alternative or supplementary strategy in conditions where the negative inotropic effects are not of primary concern,\(^6,7\) such as catecholaminergic polymorphic ventricular tachycardia (CPVT) or long QT syndrome (LQTS). However, such a strategy needs to be supported by improved understanding of the effects on Ca\(^{2+}\) homeostasis and especially of \([Ca^{2+}]_{\text{SR}}\). In the present study, we therefore aimed to establish whether LTCC antagonists could prevent Ca\(^{2+}\) waves in isolated mouse cardiomyocytes mimicking important aspects of CPVT and LQTS type 4. These conditions represent the two principle mechanisms for arrhythmogenic Ca\(^{2+}\) release: lowered threshold for SR Ca\(^{2+}\) release due to RyR mutations in CPVT,\(^8\) and excessive SR Ca\(^{2+}\) uptake due to ankyrin B mutations in LQTS.\(^9,10\) We found that \(I_{\text{calc}}\) inhibition by verapamil reduced \([Ca^{2+}]_{\text{SR}}\) and thereby decreased the incidence of Ca\(^{2+}\) waves when both the threshold for arrhythmogenic Ca\(^{2+}\) release was lowered by caffeine.\(^11\) Mimicking an important aspect of CPVT,\(^8\) and in ankyrin B heterozygous knockout mice (ankyrin B KO) with excessive \([Ca^{2+}]_{\text{SR}}\),\(^10\) mimicking LQTS type 4.\(^9\) We did not find indications of a direct effect of verapamil on RyR, an unsettled issue in previous studies,\(^12,13\) and computational modelling confirmed that lone \(I_{\text{calc}}\) is sufficient to reduce \([Ca^{2+}]_{\text{SR}}\). However, contrary to the inhibitory effect on Ca\(^{2+}\) waves, we also observed that \(I_{\text{calc}}\) inhibition was insufficient to prevent Ca\(^{2+}\) release that occurred during the decay phase of the Ca\(^{2+}\) transient, i.e. ‘early Ca\(^{2+}\) release events’.

2. Material and methods

See Supplementary material online for detailed description.

2.1 Animals and cell isolation

This investigation was approved by the Norwegian Animal Research Committee and conforms to the Directive 2010/63/EU of the European Parliament.

Male C57Bl6 and ankyrin B KO mice (8–12 weeks, 25–30 g) were used.\(^3\) Left ventricular cardiomyocytes were isolated by enzymatic digestion.\(^14\)

2.2 Experimental conditions

Beta-adrenergic receptor (\(\beta\)-AR) stimulation was induced by exposure to 0.1 \(\mu\)M isoproterenol sulphate (ISO) for a minimum of 1 min. RyR Ca\(^{2+}\) sensitivity was increased by adding 500 or 1000 \(\mu\)M caffeine to the superfusate. \(I_{\text{calc}}\) inhibition was attained with 0.5 \(\mu\)M verapamil or 1.0 \(\mu\)M nicardipine treatment for 3–6 min. All experiments were performed at 37.0°C.

2.3 Ca\(^{2+}\) measurements

Myocytes loaded with fluo-4 AM ester were superfused with a modified Heps-Tyrode’s solution A (see Supplementary material online) and field stimulated at 1 Hz. In whole-cell fluorescence experiments, Ca\(^{2+}\) release in the diastolic phase with a rate of rise slower by an order of magnitude than that of the triggered Ca\(^{2+}\) transients was counted as Ca\(^{2+}\) waves. Cells with one or more Ca\(^{2+}\) wave during the last 5 s of stimulation or within a 10 s rest period were counted as exhibiting Ca\(^{2+}\) waves. Early Ca\(^{2+}\) release events were counted as sudden increases in the fluorescence signal occurring without stimulation and before the return of the Ca\(^{2+}\) transient to baseline. Ca\(^{2+}\) sparks were measured using confocal microscopy (Zeiss LSM7 Live) in both intact and permeabilized cardiomyocytes loaded with fluo-4 AM. Cardiomyocytes were permeabilized with gramicidin (2 g·L\(^{-1}\)) and monensin (21 g·L\(^{-1}\)) for 5 min in a Ca\(^{2+}\)-free solution to investigate the effects of verapamil in the absence of functioning sarcolemmal ion channels. During these experiments, cells were exposed to a mock internal solution B (see Supplementary material online).

2.4 Field stimulation

Myocytes were field stimulated with platinum electrodes at 1 Hz by a 3 ms symmetrical bipolar pulse, 25% above threshold.

2.5 Electrophysiology

The I–V relationship for \(I_{\text{calc}}\) was determined using whole-cell voltage clamp where \(I_{\text{calc}}\) was elicited by 200 ms voltage steps from −40 mV to a range of potentials in 10 mV increments. Ten preconditioning pulses (1 Hz) from −70 to 0 mV were applied before each test pulse. \([Ca^{2+}]_{\text{SR}}\) was determined in separate experiments as the integral of \(I_{\text{ncx}}\) (\(I_{\text{ncx}}\)) elicited by 10 mM caffeine measured after steady-state \(I_{\text{calc}}\) was achieved (100 ms step pulses from −45 to 0 mV). To correct for contribution from the plasmalemmal Ca\(^{2+}\) ATPase (PMCA) to Ca\(^{2+}\) removal during caffeine release, the \(I_{\text{ncx}}\) was multiplied by a correction factor of 1.38 and 1.71 for C57Bl6 in the absence and presence of ISO, respectively, and 1.44 and 1.66 for ankyrin B KO. These factors were determined using a protocol previously described.\(^15\) Briefly, the protocol measures the rate of Ca\(^{2+}\) removal with all Ca\(^{2+}\) transporters active (Ca\(^{2+}\) transients elicited by field stimulation), with the effect of SERCA eliminated (Ca\(^{2+}\) transients elicited by 10 mM caffeine), and with both SERCA and NCX eliminated (Ca\(^{2+}\) transients elicited by 10 mM caffeine under complete NCX blockade by 10 mM Ni\(^{2+}\)). From these measurements, the relative contributions of SERCA, NCX, and PMCA to Ca\(^{2+}\) removal can be calculated. This is especially important for calculation of \([Ca^{2+}]_{\text{SR}}\) in ankyrin B KO mice, since the NCX function is likely altered in these mice.

2.6 Protein phosphorylation

The effect of verapamil on phosphorylation of key Ca\(^{2+}\) handling proteins was tested by perfusing excised hearts at 37.0°C for 2 min on a Langendorff setup with solution A containing 0.1 \(\mu\)M ISO ± 0.5 \(\mu\)M verapamil. Hearts were then removed from the cannula, and the left ventricle was dissected and immediately frozen on liquid N\(_2\). Phosphorylation status was determined using western blotting.

2.7 Computational modelling

A mathematical model was employed to study the effects of verapamil on Ca\(^{2+}\) homeostasis.\(^16\) The model includes dynamics of \([Ca^{2+}]_{\text{SR}}\), and \([Ca^{2+}]_{\text{SR}}\), as well as concentrations of other ions, and of gating variables that govern the direction and magnitude of transmembrane ion fluxes in cardiomyocytes. In the model, verapamil addition was assumed to positively affect the rate of transition from the closed active to the closed inactive state of the LTCC, whereas ISO was assumed to positively affect the opposite rate of transition as well as the affinity of the SERCA pump.

2.8 Statistics

Mean data were compared by using Student’s t-test. One-tailed comparison was employed to test the effect of verapamil on \([Ca^{2+}]_{\text{SR}}\) during ISO and caffeine exposure. Two-way repeated-measures ANOVA was used for comparison of verapamil effect on \(I_{\text{calc}}\) in the absence and presence of ISO. Fisher’s exact test was used for comparison of percentage of cells with Ca\(^{2+}\) waves in the presence and absence of verapamil. Multiple regression analysis with Ca\(^{2+}\) waves as the independent variable was employed to test the effect of verapamil, independent of ISO, caffeine and early Ca\(^{2+}\)-release events. Results are presented as mean ± standard error of mean. \(P < 0.05\) was considered statistically significant.
3. Results

3.1 \( I_{CaL} \) inhibition prevents \( Ca^{2+} \) waves elicited by \( \beta-AR \) stimulation in myocytes with increased \( Ca^{2+} \) sensitivity of RyR

We investigated the effect of \( I_{CaL} \) inhibition on \( Ca^{2+} \) wave development during \( \beta-AR \) stimulation. Action potentials were elicited by field stimulation in isolated cardiomyocytes from the left ventricle of C57Bl6 mice. The threshold for diastolic \( Ca^{2+} \) release was lowered by exposing the cells to caffeine, and \( Ca^{2+} \) waves were induced by \( \beta-AR \) stimulation with ISO (Figure 1A). \( Ca^{2+} \) waves were measured as \( Ca^{2+} \) release in the diastolic phase between \( Ca^{2+} \) transients elicited by field stimulation, and in a 10 s rest period without stimulation. \( \beta-AR \) stimulation combined with 500 \( \mu M \) caffeine induced \( Ca^{2+} \) waves in 47% of myocytes during stimulation and 93% during the rest period (Figure 1B and C, respectively). \( I_{CaL} \) inhibition with 0.5 \( \mu M \) verapamil under these conditions reduced the probability of \( Ca^{2+} \) waves by 6% (\( P < 0.05 \)) during stimulation and 21% (\( P < 0.05 \)) in the rest period. Furthermore, the frequency of \( Ca^{2+} \) waves in the rest period also decreased with verapamil (ISO + caffeine vs. ISO + caffeine + verapamil: 0.37 ± 0.12 vs. 0.02 ± 0.01 waves s\(^{-1} \), \( P < 0.05 \), Figure 1D).

The protective effect of verapamil against \( Ca^{2+} \) waves was also tested by further increasing RyR \( Ca^{2+} \) sensitivity using higher caffeine concentration (1000 \( \mu M \)). Even in these conditions, verapamil reduced the \( Ca^{2+} \) wave probability during stimulation (\( P < 0.05 \), Figure 1B) and the frequency of \( Ca^{2+} \) waves in the rest period (\( P < 0.05 \), Figure 1D). Verapamil was also effective in preventing \( Ca^{2+} \) waves in the rest period induced by ISO alone, without additional RyR sensitization with caffeine (Supplementary material online, Figure S1A–C).

To test the generalizability of our findings with verapamil, we performed similar experiments with another LTCC antagonist from a different pharmacological group, i.e. the dihydropyridine nicardipine. The preventive effect on \( Ca^{2+} \) waves in the rest period was comparable with verapamil (Supplementary material online, Figure S1E–G).

Interestingly, in all of the above experiments, we observed an additional category of abnormal \( Ca^{2+} \) release during the decay phase of the \( Ca^{2+} \) transient (Figure 1 and Supplementary material online, Figure S2). These events were termed ‘early \( Ca^{2+} \) release events’ since they could be clearly separated from \( Ca^{2+} \) waves (Supplementary material online, Figure S2A and B). The occurrence of early \( Ca^{2+} \) release showed a time-dependent increase during ISO stimulation (Supplementary material online, Figure S2C), and did not occur in voltage-clamped cells exposed to \( K^+ \)-current blockers (Supplementary material online, Figure S2D). Notably, this type of \( Ca^{2+} \) release was not prevented by \( I_{CaL} \) inhibition (Figure 1E, Supplementary material online, Figure S1D and H). In a multiple regression analysis, the occurrence of early \( Ca^{2+} \) release events did not affect the probability for \( Ca^{2+} \) waves, while verapamil had a significant negative effect on \( Ca^{2+} \) wave probability [OR for \( Ca^{2+} \) waves in the presence of verapamil 0.27 (CI 0.09–0.83), \( P = 0.02 \)].

3.2 \( I_{CaL} \) inhibition prevents \( Ca^{2+} \) wave-initiating \( Ca^{2+} \) release

\( Ca^{2+} \) waves result from a local release event, i.e. a \( Ca^{2+} \) spark, that initiates a propagating \( Ca^{2+} \) wave. Theoretically, reduced \( Ca^{2+} \) wave probability can be obtained by inhibition of the initiating \( Ca^{2+} \) sparks or by hampered wave propagation. Therefore, we investigated the effect of \( I_{CaL} \) inhibition on the initiating \( Ca^{2+} \) sparks by confocal microscopy (Figure 2). As expected, combined ISO and 500 \( \mu M \) caffeine increased the frequency of sparks compared with control conditions (\( +3030 \pm 837 \% , P < 0.05 \), Figure 2B). \( I_{CaL} \) inhibition under these conditions reduced the \( Ca^{2+} \) spark frequency by 80 ± 17% (\( P < 0.05 \)). In these experiments, \( Ca^{2+} \) waves occurred in 62% of the cells at rest after stimulation and ISO + caffeine exposure, but were completely abolished by verapamil (\( P < 0.05 \), Figure 2C). Thus, \( I_{CaL} \) inhibition prevented \( Ca^{2+} \) wave initiation.

3.3 \( I_{CaL} \) inhibition reduces \([Ca^{2+}]_{SR}\) during \( \beta-AR \) stimulation

Having established that \( I_{CaL} \) inhibition is a useful strategy for preventing \( Ca^{2+} \) waves, we further examined the underlying mechanism. Our first step in this analysis was to quantify the degree of \( I_{CaL} \) inhibition obtained by the chosen verapamil concentration. This was analysed from measurements of the current–voltage (\( I–V \)) relationship for \( I_{CaL} \) during verapamil exposure in the absence and presence of \( \beta-AR \) stimulation (Figure 3A). To mimic the conditions in the field stimulation experiments used for induction of waves, we used preconditioning pulses with voltage steps from −70 to 0 mV. As expected, \( \beta-AR \) stimulation resulted in a leftward shift of the \( I–V \) relationship, while verapamil reduced peak \( I_{CaL} \) at all voltage steps (Figure 3B). The maximal peak \( I_{CaL} \) was reduced by 40 ± 15% with verapamil in control conditions (\( P < 0.05 \)), and by 67 ± 10% during \( \beta-AR \) stimulation (\( P < 0.05 \)). The \( I_{CaL} \) inhibitory effect of verapamil was significantly larger during ISO than during control conditions (\( P < 0.05 \)).

Next, in our analysis, we studied the effect of verapamil on \([Ca^{2+}]_{SR}\). \([Ca^{2+}]_{SR}\) was measured from \( f_{N_{CaX}} \) elicited by rapid caffeine-induced SR \( Ca^{2+} \) release (Figure 3C). During control conditions, verapamil did not affect \([Ca^{2+}]_{SR}\) (Figure 3D). \( \beta-AR \) stimulation increased \([Ca^{2+}]_{SR}\) by 51 ± 14% compared with control conditions (\( P < 0.05 \)). When verapamil was added to \( \beta-AR \) stimulation, \([Ca^{2+}]_{SR}\) decreased by 20 ± 5% (\( P < 0.05 \), Figure 3D). This reduction in \([Ca^{2+}]_{SR}\) could explain the decreased probability for \( Ca^{2+} \) sparks and \( Ca^{2+} \) waves after \( I_{CaL} \) inhibition. To confirm that these findings were applicable to the conditions used for wave induction, we measured \([Ca^{2+}]_{SR}\) during combined \( \beta-AR \) stimulation and caffeine exposure. Under these conditions, verapamil reduced \([Ca^{2+}]_{SR}\) by 28 ± 8% (\( P < 0.05 \)). These findings could explain the preventive effect of verapamil on \( Ca^{2+} \) sparks and \( Ca^{2+} \) waves.

3.4 \( Ca^{2+} \) wave prevention by verapamil is not due to direct effects on RyR, nor due to altered phosphoprotein levels

Theoretically, prevention of diastolic SR \( Ca^{2+} \) release is possible through reduced \([Ca^{2+}]_{SR}\) or by increased threshold for release by stabilization of RyR. Thus, a direct blocking effect of verapamil on RyR could potentially contribute to the preventive effect on \( Ca^{2+} \) sparks and waves.12,17 We therefore examined the effect of verapamil on spontaneous release under conditions which eliminated the influence of sarcoplasmic ion channels. Permeabized cardiomyocytes were exposed to a mock internal solution and \([Ca^{2+}]_{SR}\) raised by high \( Ca^{2+} \) concentrations (300 nM) and 0.1 \( \mu M \) ISO. To avoid a potential interaction with verapamil, caffeine was not included in these experiments. Under these conditions, verapamil did not affect the
$I_{\text{CaL}}$ inhibition prevents $\text{Ca}^{2+}$ waves in myocytes with increased $\text{Ca}^{2+}$ sensitivity of RyR. (A) A field stimulation protocol was employed to test probability for $\text{Ca}^{2+}$ wave development in mouse left ventricular myocytes. Myocytes were stimulated at 1 Hz until steady state of $\text{Ca}^{2+}$ transients was reached (stimulation period). Stimulation was then stopped (rest period). During control conditions (CTR, upper panel) myocytes were exposed to a control solution A. The myocytes were then exposed to caffeine to increase the $\text{Ca}^{2+}$ sensitivity of RyR and isoproterenol (0.1 mM) to induce $\text{Ca}^{2+}$ waves (straight open arrows) (ISO + caffeine, middle panel). The effect of $I_{\text{CaL}}$ inhibition was tested by application of verapamil (0.5 mM) (ISO + caffeine + verapamil, lower panel). All tracings in (A) are from the same cell. This protocol was performed in two series of experiments with 500 and 1000 mM caffeine, respectively. (B and C) $\text{Ca}^{2+}$ wave probability is shown as fraction of myocytes that produced waves during stimulation and during the rest period, respectively. (D) Frequency of $\text{Ca}^{2+}$ waves was measured in the rest period. (E) Early $\text{Ca}^{2+}$ release events (angled closed arrows) occurred during the decay phase of the $\text{Ca}^{2+}$ transient (for further description, see Supplementary material online, Figure S2). Data from 9 mice, 17 cells with 500 mM caffeine; 7 mice, 20 cells with 1000 mM caffeine. *$P < 0.05$, paired data.
and RyR in left ventricles from hearts perfused with ISO in the presence and absence of verapamil. Verapamil did not alter phosphorylation status of either PLB or RyR (Figure 4C). Thus, alterations in PKA- or CaMKII-dependent phosphorylation did not contribute to reduced Ca\(^{2+}\) wave probability from \(I_{\text{Cal}}\) inhibition.

### 3.5 \(I_{\text{Cal}}\) inhibition is sufficient to reduce \([\text{Ca}^{2+}]_{\text{SR}}\) during \(\beta\)-AR stimulation

A mathematical model of Ca\(^{2+}\) homeostasis in ventricular cardiomyocytes was employed to further test whether the \(I_{\text{Cal}}\) inhibitory effect of verapamil is sufficient to reduce \([\text{Ca}^{2+}]_{\text{SR}}\) or whether other effects need to be assumed to account for the experimental data (Figure 5). The effect of verapamil was simulated by increasing the switch rate of the LTCC from the closed active to the closed inactive state. ISO exposure was mimicked by increasing the switch rate from inactive to closed active state and by increasing Ca\(^{2+}\) affinity of SERCA2. Parameters were chosen to reproduce \(I_{\text{Cal}}\) inhibition that was comparable with the experimental data (Figure 5A). \([\text{Ca}^{2+}]_{\text{SR}}\) was measured in a simulation of caffeine-elicited \(I_{\text{NCX}}\) equal to the method employed experimentally. \(I_{\text{Cal}}\) inhibition did not alter the \([\text{Ca}^{2+}]_{\text{SR}}\) in CTR conditions (Figure 5B, left panel). As for the experimental data, this was opposed to results during ISO exposure in which caffeine-elicited \(I_{\text{NCX}}\) was reduced after \(I_{\text{Cal}}\) inhibition (Figure 5B, right panel).

The model was then employed to test different hypothetical effects of verapamil on \([\text{Ca}^{2+}]_{\text{SR}}\) in steady state after a prolonged period of stimulation and compared with experimental data (Figure 5C). In accordance with the experimental results, \(I_{\text{Cal}}\) inhibition resulted in a decrease in \([\text{Ca}^{2+}]_{\text{SR}}\), but only in the presence of ISO [ISO (m) vs. ISO + \(I_{\text{Cal}}\) inhib. (m)].

Based on discussions in previous publications, we wanted to test whether other potential effects of verapamil could contribute. First, including decreased RyR open probability in the mathematical model did not alter the effect of \(I_{\text{Cal}}\) inhibition \([I_{\text{Cal}}\) inhib. (m) vs. \(I_{\text{Cal}}\) and RyR inhib. (m)]. This is compatible with our experiments with permeabilized cells, supporting that verapamil only has effect on LTCC in our experiments. Second, although not supported by our phosphoprotein results, reduced CaMKII activation from \(I_{\text{Cal}}\) inhibition is conceivable and could potentially contribute to our experimental results. However, when reduced CaMKII activity was added, \([\text{Ca}^{2+}]_{\text{SR}}\) decreased both in the presence and in the absence of ISO. This is in contrast to our experimental measurements of \([\text{Ca}^{2+}]_{\text{SR}}\). Third, the main regulator of \([\text{Ca}^{2+}]_{\text{SR}}\) is SERCA. We therefore tested the effect of reduced SERCA activity in our model. This addition also resulted in decreased \([\text{Ca}^{2+}]_{\text{SR}}\) both in the presence and in the absence of ISO, conflicting with the experimental results. Thus, a lone \(I_{\text{Cal}}\) inhibitory effect from verapamil was the intervention that best, and in the simplest way, reproduced our experimental findings.

### 3.6 \(I_{\text{Cal}}\) inhibition prevents Ca\(^{2+}\) waves elicited by \(\beta\)-AR stimulation in ankyrin B knockout mice with increased propensity for excessive SR Ca\(^{2+}\) accumulation

Our experiments with increased Ca\(^{2+}\) sensitivity of RyR due to caffeine exposure established that \(I_{\text{Cal}}\) inhibition prevents Ca\(^{2+}\) waves due to reduced \([\text{Ca}^{2+}]_{\text{SR}}\). This mechanism for wave prevention should be applicable even in other conditions with different mechanisms underlying increased risk of Ca\(^{2+}\) waves during \(\beta\)-AR stimulation.
Therefore, we tested the effect of verapamil on Ca\(^{2+}\) wave probability in isolated cardiomyocytes from ankyrin B KO mice. These mice have reduced expression of the anchoring protein ankyrin B, and exhibit increased propensity for DADs and triggered arrhythmias during \(\beta\)-AR stimulation.\(^9\) The mechanism for arrhythmogenic SR Ca\(^{2+}\) over-load in these mice includes reduced Ca\(^{2+}\) extrusion and a propensity for excessive Ca\(^{2+}\) accumulation in the SR during \(\beta\)-AR stimulation.\(^10\) Thus, arrhythmogenic Ca\(^{2+}\) release in cardiomyocytes from these mice is caused by a different mechanism than increased Ca\(^{2+}\) sensitivity of RyR.

We confirmed that cardiomyocytes from ankyrin B KO mice exhibit increased probability and frequency of Ca\(^{2+}\) waves, increased probability of early Ca\(^{2+}\) release events, and increased [Ca\(^{2+}\)]\(_{SR}\) during ISO exposure compared with C57Bl6 (WT) (Figure 6A–E, \(P < 0.05\) for all data). The effect of verapamil exposure was then tested in ankyrin B KO cardiomyocytes with the same protocol as used for our caffeine experiments (Figure 6F). ISO exposure resulted in Ca\(^{2+}\) waves during field stimulation in 35% of ankyrin B KO cardiomyocytes, while 69% exhibited waves during the following 10 s rest period (Figure 6G). Verapamil completely abolished Ca\(^{2+}\) waves during stimulation (\(P < 0.05\)) and reduced the probability to 29% during the rest period (\(P < 0.05\)), with a 60 \(\pm\) 15% decrease in frequency (0.20 \(\pm\) 0.04 vs. 0.08 \(\pm\) 0.03 waves s\(^{-1}\), \(P < 0.05\)). In whole-cell voltage-clamped cells exposed to ISO, verapamil reduced I\(_{CaL}\) by 71 \(\pm\) 6\% (Figure 6H). Importantly, this resulted in a 19 \(\pm\) 5\% decrease in [Ca\(^{2+}\)]\(_{SR}\) (Figure 6I). Thus, the wave preventive mechanism of verapamil found in our experiments with caffeine was also applicable to cardiomyocytes from ankyrin B KO mice.

### 4. Discussion

We have shown that I\(_{CaL}\) inhibition can prevent Ca\(^{2+}\) waves induced by \(\beta\)-AR stimulation. This strategy was effective in two different models with increased risk of Ca\(^{2+}\) waves: increased RyR Ca\(^{2+}\) sensitivity due to caffeine exposure and increased [Ca\(^{2+}\)]\(_{SR}\) due to reduced ankyrin B expression. I\(_{CaL}\) inhibition targets a common feature in all conditions where Ca\(^{2+}\) waves develop, i.e. suprathreshold [Ca\(^{2+}\)]\(_{SR}\). By lowering [Ca\(^{2+}\)]\(_{SR}\) during \(\beta\)-AR stimulation, I\(_{CaL}\) inhibition reduced the number of Ca\(^{2+}\) sparks, i.e. the initiating event in Ca\(^{2+}\) wave development. This was a direct effect of altered Ca\(^{2+}\) cycling, and not due to any effects from verapamil on RyR or phosphorylation of key Ca\(^{2+}\) handling proteins. Although effective in prevention of
Ca	extsuperscript{2+} waves, we observed that the LTCC antagonists used in our experiments did not prevent early Ca	extsuperscript{2+} release events.

4.1 Prevention of arrhythmogenic Ca	extsuperscript{2+} waves is possible with clinically relevant concentrations of verapamil

We employed verapamil to investigate the effect of I_{CaL} inhibition on arrhythmogenic SR Ca	extsuperscript{2+} waves. The verapamil concentration used is in the range of the human therapeutic plasma concentrations (80–400 ng mL	extsuperscript{-1}; 0.2–0.8 M), although plasma levels of verapamil are not routinely measured in clinical settings. I_{CaL} inhibition was ≈65% in our experiments designed to induce Ca	extsuperscript{2+} waves, as measured from I–V relations for I_{CaL} during β-AR stimulation and 0.5 M verapamil exposure. This level of inhibition reduced [Ca	extsuperscript{2+}]_{SR} sufficiently to prevent Ca	extsuperscript{2+} waves, while leaving the isolated cardiomyocytes viable and contracting. Less I_{CaL} inhibition could potentially have little effect on [Ca	extsuperscript{2+}]_{SR} since autoregulation of Ca	extsuperscript{2+} homeostasis in cardiomyocytes is highly efficient, preventing steady-state alterations over a broad range of perturbations. On the other hand, a larger inhibition could prevent Ca	extsuperscript{2+} cycling completely. Intriguingly, a 30% reduction in I_{CaL} was sufficient to prevent arrhythmias in a mathematical model of myocytes with a CPVT-associated RyR mutation (RyR

4.2 I_{CaL} inhibition prevents suprathreshold [Ca	extsuperscript{2+}]_{SR}

Reduced [Ca	extsuperscript{2+}]_{SR} from I_{CaL} inhibition may at first seem intuitive. However, direct experimental evidence is lacking. Trafford et al. reduced I_{CaL} by exposing myocytes to low external Ca	extsuperscript{2+} concentrations (0.2 mM). Paradoxically, this resulted in an increase in [Ca	extsuperscript{2+}]_{SR}. The authors concluded that the trigger function of I_{CaL} (being the trigger in Ca	extsuperscript{2+}-induced Ca	extsuperscript{2+} release) was affected more than the loading function (I_{CaL} being the main source of Ca	extsuperscript{2+} influx to the cell). Clearly, reduced I_{CaL} from decreased external Ca	extsuperscript{2+} concentrations is different from I_{CaL} inhibition by LTCC antagonists, since reduced external Ca	extsuperscript{2+} would primarily reduce the driving force for Ca	extsuperscript{2+} through the channel. Interestingly, a more recent study on CSQ

4.3 The Ca	extsuperscript{2+} wave preventive effect of verapamil depends on an intact sarcolemma

A direct effect from verapamil on RyR has been suggested since it was found that verapamil could bind directly to RyR in skeletal muscle.
Figure 5: Computational modelling finds lone I_{CaL} inhibition sufficient to reduce [Ca^{2+}]_{SR} during β-AR stimulation. A computational model of mouse cardiac myocytes published by Li et al.\textsuperscript{16} was employed to test whether lone I_{CaL} was sufficient to reduce [Ca^{2+}]_{SR} during β-AR stimulation, or whether additional effects of verapamil were needed to reproduce our experimental results. (A) The effect of verapamil was simulated by increasing the transition rate of the LTCC from the closed active to the closed inactive state. Parameters were set to reproduce the level of I_{CaL} inhibition found in our voltage clamp experiments. (B) The verapamil simulation was then employed to simulate the protocol used for measurements of [Ca^{2+}]_{SR} from caffeine-elicited I_{NCX}. This reproduced a reduction in [Ca^{2+}]_{SR} during β-AR stimulation (ISO), and no effect in the absence of ISO. (C) The model was then run for a period of stimulation until a steady state was reached after addition of I_{CaL} inhibition. The effect of lone I_{CaL} inhibition and additional hypothetical effects of verapamil were compared with experimental data. [Ca^{2+}]_{SR} was normalized to CTR or ISO in the absence of I_{CaL} inhibition for experimental and modelling data, respectively. (e) Experimental data, (m) model results. *P < 0.05.
**Figure 6** \( I_{\text{CaL}} \) inhibition prevents \( \text{Ca}^{2+} \) waves elicited by \( \beta \)-AR stimulation in ankyrin B knockout mice. The effect of verapamil on \( I_{\text{CaL}}, [\text{Ca}^{2+}]_{\text{SR}}, \) and \( \text{Ca}^{2+} \) waves was tested in left ventricular myocytes from ankyrin B KO mice. (A) ISO (0.1 \( \mu \)M) induced \( \text{Ca}^{2+} \) waves (straight open arrows) and early \( \text{Ca}^{2+} \) release events (angled closed arrows) in field stimulated (1 Hz) cardiomyocytes from ankyrin B KO and C57Bl6 wild-type (WT) mice. (B) \( \text{Ca}^{2+} \) wave probability and (C) frequency as well as (D) early \( \text{Ca}^{2+} \) release events and (E) \([\text{Ca}^{2+}]_{\text{SR}}\) are shown normalized to WT. Field stimulation experiments in B-D included 14 mice in each group, 39 WT, and 48 ankyrin B KO cells. (F) The effect of verapamil (0.5 \( \mu \)M) on \( \text{Ca}^{2+} \) wave probability in ankyrin B KO myocytes was tested in a field stimulation protocol identical to the one used for experiments with caffeine. (G) Effect of verapamil on probability and (H) frequency of \( \text{Ca}^{2+} \) waves in ankyrin B KO myocytes. Data from 14 mice, 50 (CTR), 48 (ISO), and 17 (ISO + verapamil) cells, respectively. (I) The effect of verapamil on \( I_{\text{CaL}} \) and (J) \([\text{Ca}^{2+}]_{\text{SR}}\) was measured in whole-cell voltage clamp experiments with ankyrin B KO myocytes. Mean data from 4 mice, 9 cells. * \( P < 0.05 \).
However, Galimberti and Knollmann found that a very high concentration of verapamil (>20 μM) was needed to inhibit Ca\(^{2+}\) sparks in permeabilized cardiomyocytes.\(^{17}\) We found that 0.5 μM verapamil did not affect Ca\(^{2+}\) spark frequency in permeabilized cells, indicating that the effects of verapamil seen in our experiments depend on an intact sarcolemma, and were not due to direct effects on RyR. Indeed, in cells with intact sarcolemma and functioning LTCCs, we found a decrease in spark frequency. We therefore conclude that a direct effect on RyR did not contribute to the preventive effect of verapamil in our study. This is in accordance with results in a previous study,\(^{26}\) and was supported by our computational modelling, which showed that lone I\(_{\text{CaL}}\) inhibition is sufficient to reduce \([\text{Ca}^{2+}]_{\text{SR}}\) in the presence of ISO.

### 4.4 Limitations and clinical perspectives

The focus in this study is strictly experimental, aiming to further understand Ca\(^{2+}\) cycling during Ca\(^{2+}\) wave development and explore potential therapeutic targets. In a clinical perspective, three features of I\(_{\text{CaL}}\) inhibition in general and by verapamil specifically are important. First, LTCC antagonists have a potentially negative inotropic effect. Therefore, we have limited our study to models mimicking conditions with preserved contractility. Secondly, verapamil is primarily a ‘use-dependent’ LTCC antagonist relying on activation of the channel for binding. Therefore, with increased activation frequency occurring with β-AR stimulation in vivo, one could speculate that the inhibitory effect of verapamil would be even more pronounced than in our study where cells were paced at 1 Hz. As discussed above, the level of I\(_{\text{CaL}}\) inhibition needed to obtain antiarrhythmic effects, yet still avoid contractile impairment, is uncertain. Thirdly, verapamil has been shown to also inhibit K\(^{+}\)-currents.\(^{27}\) Although the impact on human ventricular cardiomyocytes is uncertain, this aspect needs to be addressed in order to determine what conditions that might be suitable for I\(_{\text{CaL}}\) inhibition as antiarrhythmic therapy. This will be especially important for LQTS caused by K\(^{+}\)-channel mutations, as the differential distribution of I\(_{\text{CaL}}\) throughout the myocardium could predispose to reentry circuits.\(^{28}\)

A further potential limitation to the clinical use of verapamil and nicardipine is that these drugs did not prevent early Ca\(^{2+}\) release events. Such events occurred to a similar extent both in the absence and in the presence of I\(_{\text{CaL}}\) inhibition (Supplementary material online, Figures S1D and H, and S2C). Therefore, their occurrence during exposure to verapamil or nicardipine is not an effect of these drugs.

Although our experiments were not designed to address the mechanism underlying the early Ca\(^{2+}\) release events, they could be clearly separated from the diastolic Ca\(^{2+}\) waves that were the focus of our study. Our confocal microscopy linescan images clearly show that these events do not occur as propagating waves, but rather as homogeneous increases in \([\text{Ca}^{2+}]\) across the cell (Supplementary material online, Figure S2). Furthermore, the fact that the occurrence of early Ca\(^{2+}\) release events was not correlated with the occurrence of Ca\(^{2+}\) waves suggests that these events have different underlying mechanisms. Indeed, if early Ca\(^{2+}\) release events resulted from supra-threshold \([\text{Ca}^{2+}]_{\text{SR}}\) and SR Ca\(^{2+}\) release in a manner similar to Ca\(^{2+}\) waves, one would expect their occurrence to lower the \([\text{Ca}^{2+}]_{\text{SR}}\) and thereby reduce the probability for Ca\(^{2+}\) waves. Rather, the fact that early Ca\(^{2+}\) release events did not occur in voltage-clamped cells with blocked K\(^{+}\)-currents could indicate that they are caused by altered action potential configuration during verapamil and nicardipine exposure. Indeed, these Ca\(^{2+}\) release events resemble Ca\(^{2+}\) release events associated with early afterdepolarisations (EADs).\(^{29,30}\) One could speculate that the effect of verapamil, either directly on the LTCC or other ion channels, could prolong the action potential, thereby contributing to EADs. Again, however, we can only conclude that verapamil and nicardipine did not prevent the early Ca\(^{2+}\) release events. EADs can originate from both Ca\(^{2+}\) waves and reactivation of LTCC.\(^{29,30}\) Our results could indicate that I\(_{\text{CaL}}\) inhibition only prevents one of these mechanisms for triggered arrhythmias. This observation merits further studies on the effect of common LTCC antagonists on action potential configuration and other currents in addition to I\(_{\text{CaL}}\) in conditions with increased risk of triggered arrhythmias.

### 4.5 Conclusions

I\(_{\text{CaL}}\) inhibition with verapamil prevented arrhythmogenic Ca\(^{2+}\) waves elicited by β-AR stimulation in diastole, but did not prevent unstimulated Ca\(^{2+}\) release events occurring during the decay phase of the Ca\(^{2+}\) transient. I\(_{\text{CaL}}\) inhibition prevented diastolic Ca\(^{2+}\) waves caused by increased Ca\(^{2+}\) sensitivity of RyR and by excessive SR Ca\(^{2+}\) accumulation due to reduced ankyrin B abundance. The preventive effect could be explained in full by reduced \([\text{Ca}^{2+}]_{\text{SR}}\) resulting from I\(_{\text{CaL}}\) inhibition. This effect of LTCC antagonists could prove beneficial for prevention of triggered arrhythmias in conditions with normal contractile function such as CPVT and LQTS type 4. However, the lack of effect on Ca\(^{2+}\) release events occurring during the decay phase of the Ca\(^{2+}\) transient limits such clinical use until the mechanism of these events and the Ca\(^{2+}\) waves, as well as their propensity to trigger arrhythmias, are better understood.

### Supplementary material

Supplementary material is available at Cardiovascular Research online.

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### Conflict of interest

None declared.

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