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Ca^{2+} leak, what is it? Why should we care? Can it be managed?

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Ca^{2+} leak, what is it? Why should we care? Can it be managed?

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ABSTRACT

For arrhythmia triggers that are secondary to dysfunctional intracellular Ca^{2+} cycling, there are few if any specific agents that target exactly the Ca^{2+} handling machinery. However, in the literature to date, several candidates have been proposed. We review here these agents with the idea that in the future these agents or those derived thereof will prove invaluable in clinical application.

INTRODUCTION

Under normal conditions for all cardiac cells, during systole, Ca^{2+} influx through the cardiac calcium channel provides a trigger for the calcium to be released from the sarcoplasmic reticulum (SR) through a large SR membrane, ligand operated, ion channel called the ryanodine receptor (RYR). The open probability of the RYR protein is increased by the elevation of cytoplasmic Ca^{2+} concentration $[Ca^{2+}]_i$. Thus, Ca^{2+} entry into the cell produces a small increase of Ca^{2+} which leads to an opening of the RYR and subsequent release of a larger amount of Ca^{2+} that is stored in the SR. This process is known as calcium induced calcium release (**CICR) (Figure 1)**. Microscopic signals resulting from clusters of RYR openings generate Ca^{2+} signals called Ca^{2+} sparks. Spatial and temporal summation of action potential evoked Ca^{2+} sparks underlies the global Ca^{2+} transient which in contractile cells has a familiar rise and decay as Ca^{2+} is released is reuptaken into the SR ready for the next heartbeat. Any remaining cytosolic Ca^{2+} is pumped out of cell by sodium calcium exchanger protein (NCX). Under normal conditions, CICR that occurs does not propagate but rather remains controlled by L type Ca^{2+} channel influx.

So what is "Ca^{2+} leak" if the cell always has spontaneous Ca^{2+} sparks, albeit at low probability?

When a cell is "overloaded" with calcium the associated sequestration of Ca^{2+} by the SR can increase SR Ca^{2+} content to above normal levels, under these circumstances the Ca^{2+} leaks out of the SR in the form of Ca^{2+} waves. These are local Ca^{2+} release events that trigger a regenerative Ca^{2+} waves via the CICR process. The Ca^{2+} wave can propagate throughout the cell and in some cases can trigger a Ca^{2+} waves in an adjacent cell (**Figure 2**)(see also [1]). It appears that intracellular Ca^{2+} waves generally

occur when the SR Ca^{2+} content is elevated above a threshold value [2, 3], but other changes, such as altered Ca^{2+} sensitivity of the RyR can induced Ca^{2+} waves. Some of the Ca^{2+} in the wave is pumped out of the cell by the electrogenic NCX. The resulting current depolarizes the membrane (producing a delayed afterdepolarization (DAD) like membrane voltage change) and can be sufficient to initiate an action potential. Yet synchrony of Ca^{2+} releases between coupled cells is required for to provide sufficient depolarizing current within one region to initiate an arrhythmic action potential in an intact ventricle/atrium. The critical number of coupled cells experiencing a DAD is a topic of debate and research [4-6].

SR Ca^{2+} leak is increased in numerous pathological conditions (eg. Heart failure (HF) [7];post MI [8, 9]). SR Ca^{2+} leak, if persistent, decreases SR Ca^{2+} load and as explained above can lead to propagating Ca^{2+} waves and thus DADs (**Figure 3**).

While Ca^{2+} leak is an operational term, several mechanisms have been proposed to explain the altered RYR gating that leads to Ca^{2+} leak. An increased sensitivity of RYR to its ligand cytosolic Ca^{2+} may be due to enhanced protein kinase A (PKA) and/or CaMKII dependent RYR [7] phosphorylation at specific sites [10 11-13]. Recent data using human tissues favor one idea where Ca^{2+} handling abnormalities in HF are due to excessive CaMKII phosphorylation at a specific RYR residue [14 15]. Other factors such as the oxidative state could change resulting in direct activation of the RYR protein[16]. Finally others have suggested that RYR gating may be altered when an abundance of endogenous proteins that modulate RYR are altered (eg. sorcin, S100A [17, 18]).

Finally mutations and dysregulation of RYR and other calcium binding proteins have been implicated in several gene-based arrhythmias; for example, RYR and CVPT, and Calsequestrin(CASQ) and CPVT [19]. Mechanisms of these arrhythmias are similar to those of acquired diseases above, that is the arrhythmic events are caused by

abnormally propagating Ca^{2+} waves which cause NCX dependent membrane oscillations (DADs) and triggered beats (**Figures 2,3**).

Can Ca^{2+} leak be Managed?

As an antiarrhythmic, we would want an agent to modulate the mishandled Ca^{2+} so Ca^{2+} does not increase Ca^{2+} dependent currents to cause depolarization and elicit action potentials. If we target the spontaneous Ca^{2+} releases, then we would reduce the initiators of the Ca^{2+} waves, the delayed afterdepolarizations and thus triggering beats.

The arrhythmias mentioned above result when the cell's SR Ca^{2+} content is increased above a threshold level at which waves are produced. Recent work suggests that a decrease of threshold (due to a sensitization to RYR Ca^{2+} release) also produces Ca^{2+} waves and DADs. For arrhythmias seen in heart failure the involvement of DADs in some ventricular arrhythmias has been shown [20, 21]. However, in heart failure the SR Ca^{2+} content is decreased suggesting that the threshold for Ca^{2+} release may be lower, such that Ca^{2+} waves would occur at a lower SR Ca^{2+} content. This may be a consequence of increased leakiness of the RYR during diastole, such that there is increased Ca^{2+} efflux at a given SR Ca^{2+} content. The exact molecular mechanisms responsible for this are controversial [22], but as above, it may be associated with increased phosphorylation of the RYR due to PKA or CaM-Kinase [14].

An example of the occurrence of DADs in the absence of increased SR Ca^{2+} content is provided by catecholaminergic polymorphic ventricular tachycardia (CPVT). This arrhythmia in patients is seen during exercise or other stress. The similarity of the abnormalities in the ECGs to those observed in digitalis toxicity led to the suggestion of similarities in an underlying mechanism. Genetic studies have shown that many CPVT patients have a mutation in RYR (eg R4496C) or the intrasarcoplasmic protein CASQ

(eg. R33Q). The current hypothesis is that the mutated protein causes an increased leak of Ca^{2+} from the SR. Thus Ca^{2+} waves and DADs occur at a lower SR Ca^{2+} content than in controls [23].(**Figure 3**)

Therapies for DAD-related arrhythmias

For these Ca^{2+} dependent (Ca^{2+} wave dependent) arrhythmias, the goal of therapy is to treat 1) to prevent the DAD from occurring and/or 2) to prevent the DAD from triggering an action potential.

The latter can potentially be achieved using sodium channel blockers. A better solution, however, would be to remove the underlying DAD directly. In the case of arrhythmias resulting from "calcium overload", it may be possible to remove the underlying "overload". For example, local anesthetics (eg. flecainide) reduce intracellular Na^+ concentration as a consequence of decreasing Na^+ entry (via inhibition of sodium current resulting in reduced excitability). Lowered intracellular Na+ concentration will act via NCX, to decrease intracellular Ca^{2+} load [24]. Antiarrhythmic effects of flecainide have been seen in murine models as well as patients with CPVT [25], but the confirmation that the cellular basis is linked to intracellular Na^+ levels has yet to be made.

Recently, the late Na^+ current ($I_{Na\text{-}late}$) has gained interest since it is modulated in disease. A small fraction of cardiac sodium channels carry $I_{Na\text{-}late}$. For peak I_{Na}, sodium channels open quickly and close in a well-defined time and voltage dependent manner. $I_{Na\text{-}late}$ current is formed when sodium channels remain open or reopen for 100s of ms after the peak current.

In HF and congenital long QT type3, $I_{Na\text{-}late}$ is upregulated and provides enhanced Na^+ influx during the AP [26]. This in turn alters $Ca^{2+}{}_I$ which then could be arrhythmogenic [27].

Ranolazine inhibits cardiac $I_{Na\text{-}late}$ as well as other channels (eg I_{Kr}) [28]. Some have reported it also inhibits RyR directly [29]. It has been reported to have antiarrhythmic properties in various animal models (eg HF, [30, 31]). In recent clinical trials it was demonstrated to reduce arrhythmic events [32, 33] although there appears to be a risk of Torsades de Pointes [34]. GS-967 is a newer more specific $I_{Na\text{-}late}$ current inhibitor (lacks the I_{Kr} blockade seen with Ranolazine) that shows promise as an antiarrhythmic [35, 36].

While Na^+ channel blockade of the cardiac Na^+ channel is considered now to be a viable therapy for Ca^{2+} mediated arrhythmias, new data suggest that selective blockade of Neuronal Na^+ channels (eg. Nav1.1,Nav1.3 and Nav1.6s etc) in cardiac T-tubules using riluzole is anti-arrhythmic [37]. This suggests that there is a contribution of Na^+ influx from overactive neuronal Na+ channels in RYR subcellular regions to more Ca^{2+} leak from SR (**Figure 4)**.

Theoretically, it would be possible to modulate Ca^{2+} by affecting the membrane transports/channels involved in Ca^{2+} homeostasis. For example, L type Ca^{2+} channel pore blockers obviously decrease Ca^{2+} influx and in so doing would be expected to eventually reduce SR load, Ca^{2+}_i and diminish force. Thus Ca^{2+} channel pore blockers (eg. Verapamil) will affect intracellular Ca^{2+} and wave formation but at the expense of force generation. An alternative option to drugs acting directly on molecular targets of the SR is to modulate sarcolemma Ca^{2+} influxes that in turn reduce SR Ca^{2+} load and therefore associated Ca^{2+}-leak related abnormalities. As with other targets, the risk associated with reduced SR Ca^{2+} load is that peak systolic Ca^{2+} will be reduced and associated inotropy. Currently accepted medications such as Ca^{2+} channel blockers and beta blockers reduce cardiovascular mortality partially via reduction of Ca^+ influx to the heart through their effects on the L-type Ca^{2+} channel. But the relative contribution of SR unloading to the overall beneficial effect of these two classes of drugs is difficult to assess.

Alternatively, one might target the molecular mechanism involved in the inactivation of Ca^{2+} channel proteins or the Ca^{2+} dependent processes known to affect Ca^{2+} channel function (eg. CaMKII) or small proteins (eg.Gem) that are known to affect Ca^{2+} channel subunit assembly [38].

Phosphorylation/dephosphorylation of the enzyme CaMKII is critical for cardiac excitability and function much like its well-known "neighborhood" protein, protein kinase A (PKA). Unlike PKA, CaMKII has the ability to become autophosphorylated and this is a Ca^{2+} independent process [39]. But like PKA, CaMKII activity is linked to the function of several intracellular cardiac proteins, for example, the L type Ca^{2+} channel [40] and RYR [41]. Thus targeting inhibition of this enzyme to alter function of regulated proteins to ameliorate Ca^{2+} wave function and resulting DADs is a goal of both academia and industry [42].

At this time, only three tools are available. KN-93 (and its inactive analog KN-62) are used frequently in experimental studies to illustrate the role of CaMKII in cardiac cell function. KN-93 does inhibit activation of CaMKII but not its autophosphorylation activity. But CaMKII inhibition prevents catecholamine induced VTs in CPVT mice [43] and recently has proven useful on atrial arrhythmias secondary to Ca^{2+} leak [44].

However, KN93 affects L type Ca^{2+} channel function [45] as well as some K^{+} channel function [46]. Experimentalists have also used autocamtide-3 inhibitor (AC3-1) peptides that inhibit CaMKII selectively over PKA, PKC [47]. AC3-1 is also a potent PKD inhibitor. These peptides remain as tools. There has also been a recent emergence of pharmacologically active agents designed after small endogenous proteins that inhibit CamKII, such as CaMKIIN [48] and CaMKIINide [49]. Work continues to delineate how these inhibitors affect cardiac function.

Direct modulation of SR leak via actions on RYR channel

Designing drugs to bind to RYR directly to reduce the Ca^{2+} sensitivity of the channel is thought to be a valid anti-arrhythmic strategy, but no compounds to date have been approved for clinical use purely on this mechanism. While many drugs designed for other purposes have been found to alter RYR Ca^{2+} sensitivity, these have been used as tools to investigate the effects of drug-induced modulation of RYR. The anesthetics such as tetracaine, which reduces surface membrane excitability via Na^{+} channel inhibition is also known to reduce the sensitivity of Ca^{2+} induced SR Ca^{2+} release via a direct action on RYR [50]. Studies have shown tetracaine substantially reduces the frequency of both Ca^{2+} sparks and spontaneous Ca^{2+} waves [51] but this effect is accompanied by an increased quantity of Ca^{2+} released from the SR at each spontaneous event (increased leak). Derivatives of tetracaine that block RyR appear to inhibit SR Ca^{2+} leak and prevent CPVT arrhythmias in mice [52]. Caffeine, a compound known to increase the Ca^{2+} sensitivity of RYR will increase the frequency of sparks and Ca^{2+} waves and each release event is smaller [53]. Interestingly, while these two compounds dramatically affect spontaneous Ca^{2+} release in different ways, the effect on systolic Ca^{2+} release (in the steady-state condition) is undetectable due to an autoregulatory mechanism involving Ca^{2+} influx via the L-type and Ca^{+} extrusion mainly via NCX [27]. Caffeine is known to increase ventricular premature beats in normal hearts via its ability to increase the incidence of spontaneous Ca^{2+} waves and thus generate a spontaneous diastolic depolarization which generates triggers the extra systole. Many aspects of this explanation still require clarification.

Drugs have been identified that have an almost exclusive effect on the RYR protein complex to reduce Ca^{2+} sensitivity.

The first drug candidate to emerge was the benzodiazepine derivative variously known as JTV519/K201. This molecule is similar in structure to L-type Ca^{2+} channel blockers, but was selected for its ability to reduce the effects of intracellular Ca^{2+}

overload [54]. Subsequent work suggested that the drug's mechanism was to bind to RYR and mimic the binding of the regulatory protein FK506 binding protein (FKBP12.6) [55]. FKBP12.6 is thought to bind to RYR and chronically reduce Ca^{2+} efflux through RYR. As part of the beta-adrenergic response, A-kinase mediated phosphorylation may alter the Ca^{2+} sensitivity of RYR via reduced binding of FKBP12.6. In HF, the associated altered status of the adrenergic signaling pathway in cardiac muscle is thought to result in hyper phosphorylation of RYR, reduced FKBP12.6 binding and thereby increase RYR-mediated Ca^{2+} leak from the SR, i.e. acting in an analogous way to caffeine [56]. These changes are thought to be responsible for the failing myocardium being more prone to DADs and subsequent pro-arrhythmic VPCs. In support of this, JTV519 improved outcome in an animal model of HF [57]. JTV519/K201 reduces Ca^{+} efflux via RYR at concentrations that allow reasonable specificity to RYR and appears to have an action in the absence of activation of the A-kinase pathway [58]. Therefore, regardless of the mechanism, the drug has the possibility to act via RYR as an antiarrhythmic agent on myocardium prone to arrhythmias due to dysfunctional RYR, e.g. Purkinje cells that survive in the infarcted heart [8] (**Figure 5**). An alternative structure thought to be an even more potent inhibitor of RYR is variously known as S107 or RyCal [10, 59, 60]. Others are currently being sought.

Dantrolene and dantrolene-like compounds while showing no effect on normal RyR function, inhibits Ca^{2+} leak in cells from failing hearts by promoting a stable RyR conformational state [61-63].

Action on other targets that may aid anti-arrhythmic effects: One feature of small molecules is a lack of specificity that may benefit or counteract their ability to suppress spontaneous Ca^{2+} release. For example, JTV519/K201 was found to inhibit SERCA activity by a small amount (~10%) at drug levels that also significantly suppress RYR

activity [58]. Suppression of SERCA is normally associated with smaller systolic Ca^{2+} releases and poor contractility in failing myocardium. Thus low levels of inhibition may not have the significant negative inotropic effects but could significantly suppress spontaneous Ca^{2+} waves and therefore arrhythmias. Data from several groups [64-66] suggest that a burst of Ca^{2+}-activated SERCA activity in regions of a cell adjacent to a region of spontaneous Ca^{2+} release could locally enhance SR load and increase the chance of spontaneous Ca^{2+} release propagating along the length of the cells. Thus mild SERCA inhibition may aid an anti-arrhythmic action through this route.

Studies on a CPVT mouse model and a limited number of human CPVT patients have shown that Flecainide (a Na^+ channel blocker) can suppress arrhythmias associated with RYR dysfunction [67 68]. The study suggests that the mode of anti-arrhythmic action is not via Na^+ channel inhibition, but via an inhibitory effect of flecainide on RYR [69]. But this interpretation of flecainide's action on RYR has been challenged by isolated RYR studies [70] and in intact cell work [71]. Another example of a potential revision of the mode of action of a cardiovascular drug is the beta blocker Carvedilol. This drug is a potent inhibitor of RYR and may act to suppress arrhythmias through this route [72]. Further screens of beta-blockers have identified other examples of drugs that suppress RYR activity and therefore potentially possess anti-DAD and antiarrhythmic activity [73]. Such examples indicate the challenges in designing anti-arrhythmic therapy around a single target with a single molecule and in assigning mechanism to the observed anti-arrhythmic effect. However, in a recent publication, derivatives of tetracaine with high specificity to RyR were found to effectively suppress arrhythmias in a mouse model of CPVT [52] and indicates that drug-based RyR inhibition can have powerful anti-arrhythmic effects.

Alternative anti-arrhythmic strategies:

Alternative approaches being considered are for example, inhibition of the NCX. This may appear a good strategy since this exchanger provides the major Ca^{+} activated currents(I_{ti}) in DADs [73, 74]. But tonic inhibition of this exchanger will reduce the Ca^{2+} efflux capacity of the myocardium, increase intracellular Ca^{2+} and SR load and potentially increase the probability of pro-arrhythmic Ca^{2+} release. A second approach that may indirectly be anti-arrhythmic is to use novel drugs designed to stimulate the sarcolemmal Na^{+}/K^{+} pump/antiporter (NKA) [75]. These are designed to restore the intracellular Na^{+} concentration and in doing so, may reduce cellular Ca^{2+} load via NCX, SR load and associated pro-arrhythmic SR Ca^{2+}-leak.

In summary, pharmacological strategies that specifically address abnormal SR Ca^{+} leak are at early stages of development but hold great promise as a means of providing novel anti-arrhythmic therapeutic options for a range of cardiac pathologies with associated high risk of sudden arrhythmic cardiac death.

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FIGURE LEGENDS

Figure 1 - **Simple diagram of the excitation-contraction coupling system in the cardiac cell**. During the action potential Ca^{2+} enters the cell as a rapid influx followed by a maintained component of the slow inward Ca^{2+} current (Thick arrow). The rapid influx of Ca^{2+} via the T tubules is thought to induce release of Ca^{2+} from a release compartment in the SR, by triggering opening of Ca^{2+} channels via binding sites(sensors) on RYR protein. Relaxation follows when the cytosolic Ca^{2+} is sequestered again in an uptake compartment of the SR (SERCA pump, green boxes) and partly extruded through the cell membrane by the Na^{+}/Ca^{++} exchanger (NCX). The process of NCX is electrogenic so that Ca^{2+} extrusion through NCX leads to a depolarizing current. From Ter Keurs and Boyden, Physiol Review, 2007 [76].

Figure 2. Representative confocal line-scan images show spontaneous Ca^{2+} release events (SCaEs) in wild-type (WT) and R33Q (CPVT mutation in CASQ) cells in the presence of isoproterenol. Black arrows indicate field stimulations. Spontaneous Ca events(SCaEs) in WT myocytes were usually due to a cell-wide wave that was initiated at 1 site (red arrow). SCaEs in diseased R33Q cells varied. Often, fragmented spontaneous Ca^{2+} waves occurred and slowly propagated (cells 1 and 2), and wavelets and Ca^{2+} sparks occurred before Ca^{2+} transients resume the diastolic level. From Liu N et al. Circulation Research 2013;113:142-152 [77].

Figure 3. Action potentials recordings in a R33Q mouse cells in the presence of isoproterenol at 1- to 3-Hz pacing. Early afterdepolarizations occurred at lower pacing frequency; diverse patterns of action potential were shown in all pacing frequencies. Bottom, The enlarged membrane oscillations occurring between stimulated beats. From Liu N et al. Circulation Research 2013;113:142-152 [77].

Figure 4-*Schematic diagram of a t-tubule and associated junctional SR*. Microfolds in t-tubule are depicted based on recent findings. Different arrangements of Ca^{2+} cycling proteins and sodium channels are depicted along the t-tubule. Regions highlighted by

the dashed boxes are presented at higher magnification in *C* and *D*. Note that differential shading of the interstitial space within the t-tubule and the cytoplasm within the dyadic cleft indicates local differences in ionic concentrations within these spaces due to their diffusional isolation from the bulk interstitial space and cytoplasm, respectively. *B*, results from Duolink assays (PLAs) show close association of nNaV isoform $Na_V1.6$ with both RYR2 and NCX throughout myocytes, consistent with enrichment of nNa_Vs in t-tubules. In contrast, PLA signal corresponding to association between cNa_V ($Na_V1.5$) and RYR2 is only observed at the periphery of the cell, consistent with cNa_V localization at the lateral membrane. Adapted from Radwański *et al*. 2016, doi:10.1016/j.jacbts.2016.04.004, Creative Commons Attribution-NonCommercial-No Derivatives License (CC BY NC ND). *C*, higher magnification views of regions from *A* showing two possible scenarios of nNa_V localization within t-tubules. Left, case 1, very close association between nNa_Vs and RYRs, which is consistent with PLA results. A cNaV is depicted faded since experimental results including PLA results argue against cNa_V enrichment in t-tubules. Right, case 2, nNa_Vs localized to t-tubules but not very closely associated with RYRs, which is *not* consistent with PLA results. *D*, higher magnification view of region from *A* showing cNa_V ($Na_V1.5$) localization at the lateral membrane. From Veeraraghavan et al, The Journal of Physiology, © 2017 The Physiological Society. [37] Reproduced by permission of John Wiley and Sons, Inc.

Figure 5- JTV519(K201) suppresses cell wide Ca waves in Purkinje cells from the infarcted heart A, Graph showing the incidence of cell wide Ca^{2+} waves in Normal Zone Purkinje Cells(NZPCs) and Infarct Zone Purkinjes (IZPCs) in the absence and presence of JTV519 (K201) 1 μmol/L (gray bar). B, Ca event rate, spatial extent, and amplitude in IZPCs in the absence and presence of JTV519 (K201) (gray bars). Total number of events used is shown in parentheses. From Hirose M et al. Circ Arrhythm Electrophysiol 2008;1:387-395 [8].

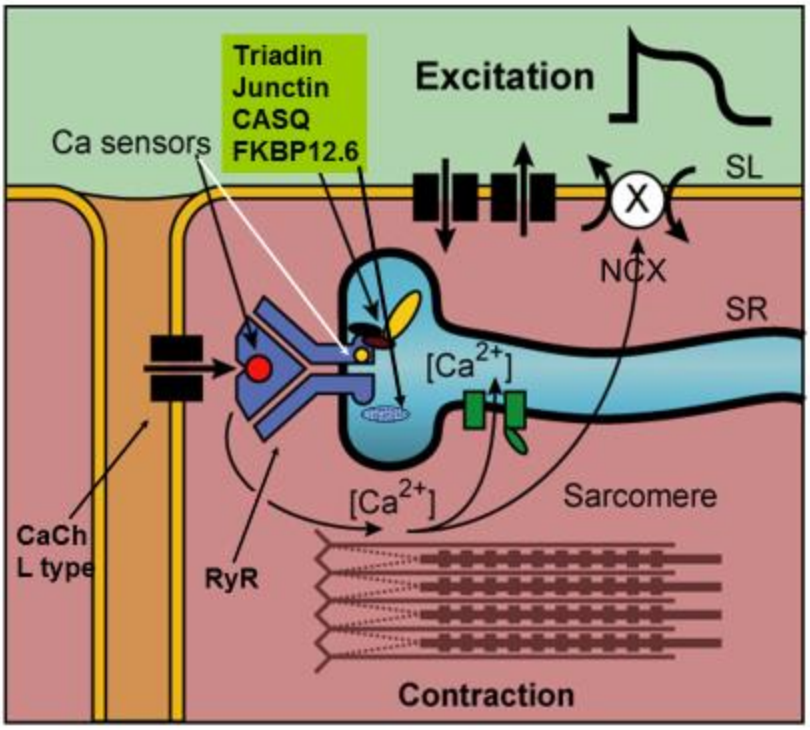


FIGURE 1

Figure 2

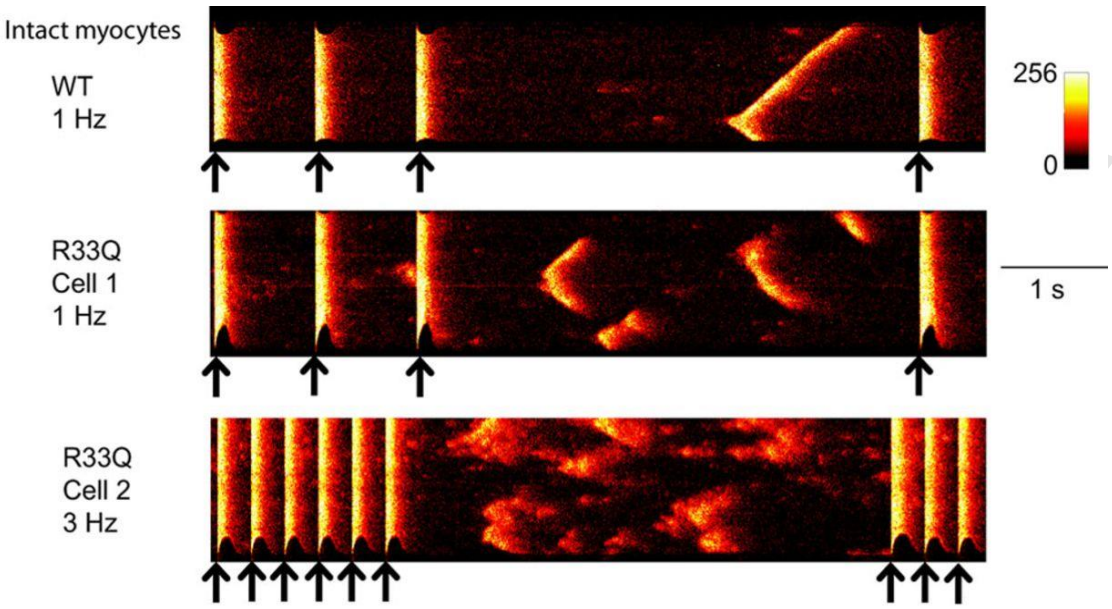


FIGURE 3

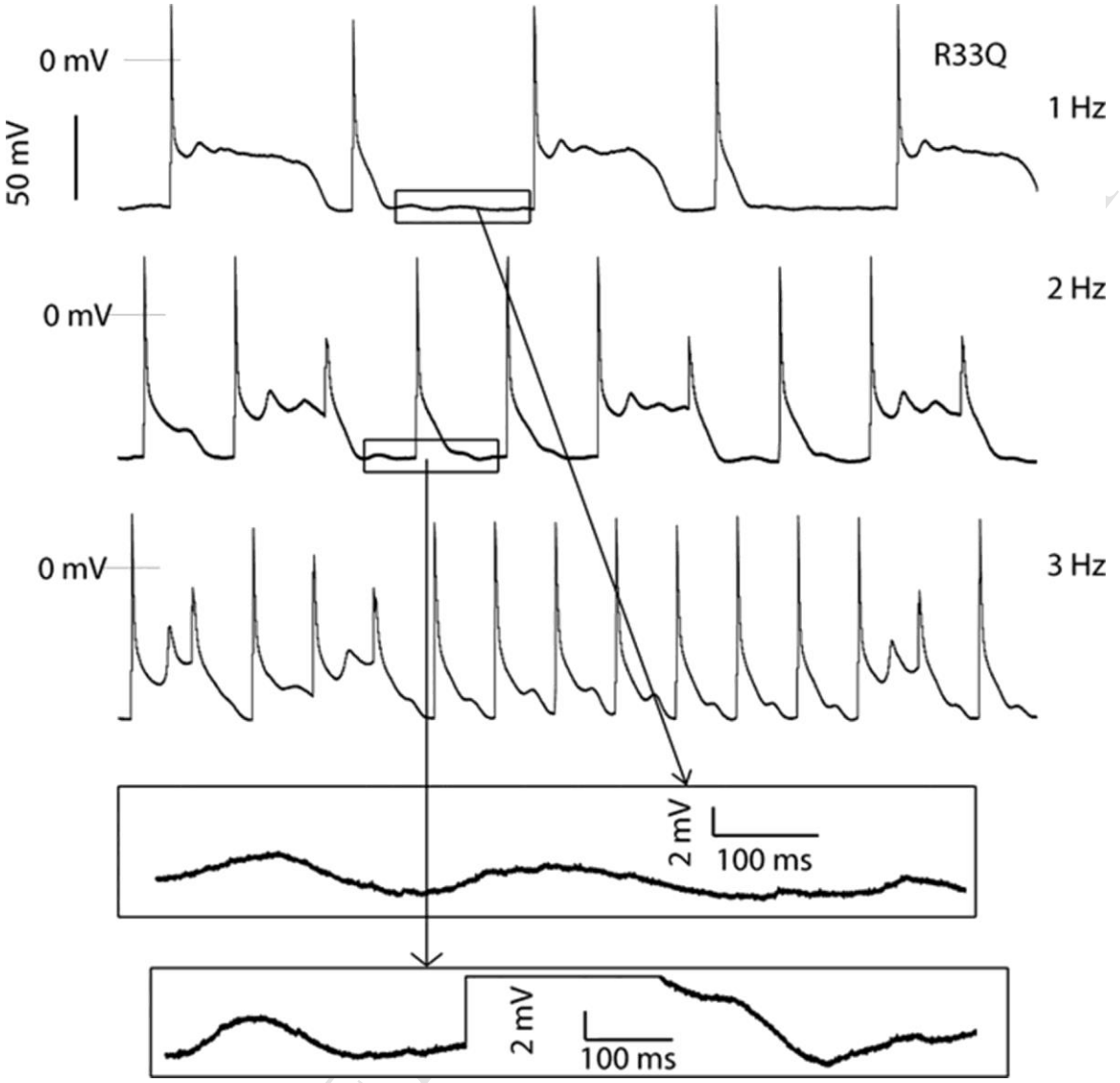


Figure 4

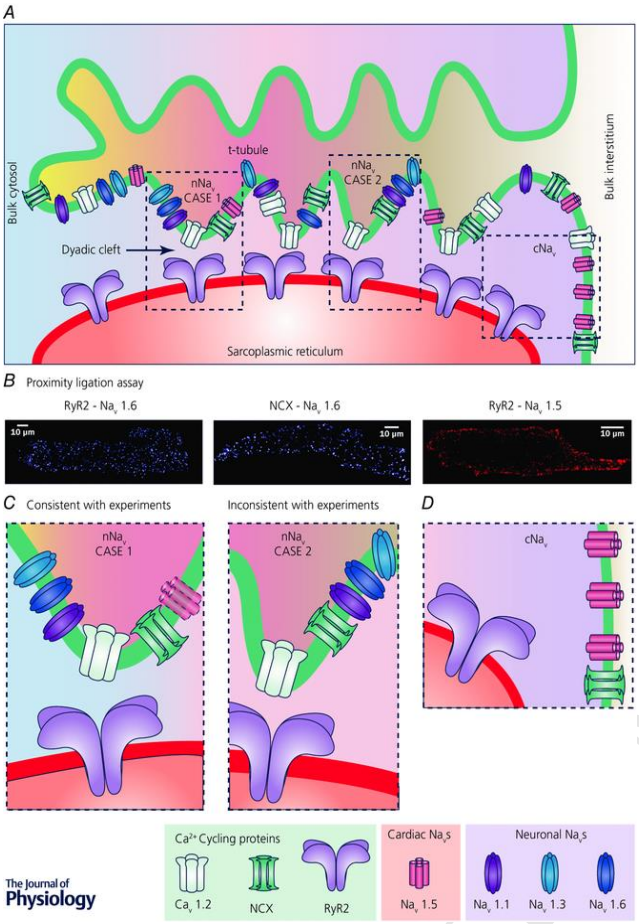
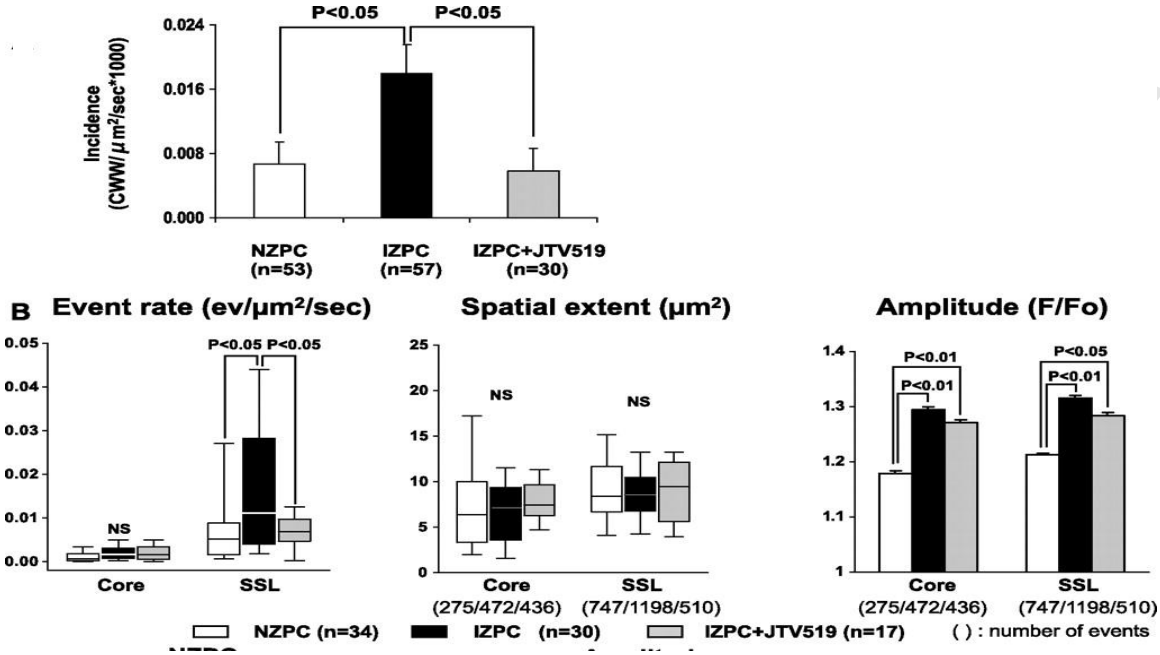


Figure 5


P<0.05
P<0.05
Incidence (CWW/μm²/sec*1000)
0.024
0.016
0.008
0.000
NZPC (n=53)
IZPC (n=57)
IZPC+JTV519 (n=30)
B
Event rate (ev/μm²/sec)
Spatial extent (μm²)
Amplitude (F/Fo)
P<0.05 P<0.05
NS
Core
SSL
NS
NS
Core
SSL
(275/472/436)
(747/1198/510)
P<0.01
P<0.01
P<0.05
P<0.01
Core
SSL
(275/472/436)
(747/1198/510)
NZPC (n=34)
IZPC (n=30)
IZPC+JTV519 (n=17)
( ) : number of events