The ryanodine receptor channel as a molecular motif in atrial fibrillation: pathophysiological and therapeutic implications

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Atrial fibrillation (AF) is the most common cardiac arrhythmia and is associated with substantial morbidity and mortality. It causes profound changes in sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} homeostasis, including ryanodine receptor channel dysfunction and increased propensity to atrial arrhythmias. In this review, we will focus on the molecular basis of ryanodine receptor channel dysfunction in AF. The potential relevance of increased incidence of spontaneous SR Ca\textsuperscript{2+} release for both AF induction and maintenance and the development of novel mechanism-based therapeutic approaches will be discussed.

Keywords
Calcium handling • Atrial fibrillation • Remodelling • Ryanodine

Abstract
Atrial fibrillation (AF) is the most common cardiac arrhythmia and is associated with substantial morbidity and mortality. It causes profound changes in sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} homeostasis, including ryanodine receptor channel dysfunction and increased propensity to atrial arrhythmias. In this review, we will focus on the molecular basis of ryanodine receptor channel dysfunction in AF. The potential relevance of increased incidence of spontaneous SR Ca\textsuperscript{2+} release for both AF induction and/or maintenance and the development of novel mechanism-based therapeutic approaches will be discussed.

1. Introduction
Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia and is associated with increased cardiovascular morbidity and mortality. Current therapeutic approaches have major limitations, including low efficacy and enhanced risk of proarrhythmic events in the ventricle. The major cause of increased morbidity in AF patients is stroke due to thromboembolism, which arises from blood stasis in atria as a consequence of decreased atrial contractility. Impaired intracellular Ca\textsuperscript{2+} handling is believed to play a critical role in atrial mechanical dysfunction. The smaller increase of intracellular Ca\textsuperscript{2+} during systole (i.e. the systolic Ca\textsuperscript{2+} transient) is a major cellular determinant of contractile dysfunction in AF. Moreover, susceptibility to spontaneous diastolic sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release through ryanodine receptor channels (RyR2) appears higher in AF and might contribute to AF-associated arrhythmogenesis by promoting both triggered activity and/or reentry. This review focuses on the molecular basis of altered RyR2 function in AF, particularly on defective phosphorylation that causes SR Ca\textsuperscript{2+} leak and discusses possible consequences of increased SR Ca\textsuperscript{2+} leak for AF promotion and/or maintenance. For discussions of other important aspects of RyR2 regulation (e.g. competitive binding of Mg\textsuperscript{2+} or stretch) or modelling of RyR2 function, the readers are referred to recent pertinent articles.

2. Fundamental mechanisms of AF: reentry vs. triggered activity
Two major mechanisms may cause AF at the organ level: reentry and ectopic activity (Figure 1). The sources underlying these mechanisms are often localized in one of the pulmonary veins or in the left-atrial posterior wall. Risk factors (i.e. age or gene mutations) and co-morbidities (i.e. heart failure or hypertension) predispose to AF by causing specific electrical and structural changes (remodelling) that produce diverse arrhythmogenic substrates, promoting arrhythmia maintenance. The homeostatic responses to the high-atrial rate rapidly cause remodelling of atrial repolarization, promoting reentry, and AF perpetuation. Concomitant cardiovascular diseases can also provide the trigger for AF initiation (e.g. acute atrial dilatation). Thus, the substrate caused by risk factors and concomitant disease conditions preceding AF is the key for AF perpetuation.

In addition to a susceptible substrate, reentry requires a trigger, usually provided by an ectopic beat (Figure 1). Ectopic activity may contribute to AF initiation by acting as a trigger of reentry, but persistence of ectopic activity may also sustain AF by facilitating reentry. Ectopic activity is caused by abnormal spontaneous discharges that can result from oscillations of the myocyte membrane potential (afterdepolarizations). Afterdepolarizations may occur during repolarization [early
afterdepolarizations (EADs)) or after completion of the action potential delayed afterdepolarizations (DADs). Left-atrial sources of ectopic activity appear to be of particular importance in a subset of patients with paroxysmal AF. EADs occur during excessive action potential (AP) prolongations and are commonly associated with bradycardia or pauses. DADs typically result from SR Ca$^{2+}$ leak due to spontaneous (non-synchronized) diastolic SR Ca$^{2+}$ release, which is caused by either SR Ca$^{2+}$ overload, RyR2 dysfunction, or a combination of both. With confocal microscopy, SR Ca$^{2+}$ leak has been visualized as Ca$^{2+}$ sparks, but invisible non-spark release events may also occur. Diastolic SR Ca$^{2+}$ release manifest as either individual or series of small-amplitude membrane-voltage oscillations that could also lead to triggered APs. In diseased hearts, spontaneous SR Ca$^{2+}$ release through RyR2 activates a transient inward current ($I_{ti}$), which is largely carried by Na$^{+}$/Ca$^{2+}$ exchanger (NCX) and is the dominant contributor to induction of cellular DADs. Potentially arrhythmogenic DADs have been demonstrated in isolated diseased human atrial appendages. AF-related remodelling increases the likelihood of atrial cellular DADs, which is described subsequently.

### 3. Ca$^{2+}$-signalling properties in normal atria

As in ventricular myocytes, excitation-contraction coupling in atrial myocytes starts with Ca$^{2+}$ entry via voltage-gated L-type Ca$^{2+}$-channels ($I_{Ca,L}$) that triggers a greater SR Ca$^{2+}$ release via RyR2, a process known as Ca$^{2+}$-induced Ca$^{2+}$ release. However, in the absence of a fully developed T-tubule system in atria, Ca$^{2+}$ influx triggers a sequential (non-synchronous) increase in [Ca$^{2+}$], whereby Ca$^{2+}$ waves start in the atrial-myocyte periphery (junctional SR) and then propagate to the myocyte centre, recruiting additional Ca$^{2+}$-releasing sites. The size of the systolic Ca$^{2+}$ transient depends on both open probability of RyR2 and SR Ca$^{2+}$ content, which is indirectly a function of Ca$^{2+}$ reuptake through SR Ca$^{2+}$-ATPase (SERCA2a). Removal of cytosolic Ca$^{2+}$ during diastole occurs primarily by SERCA2a-mediated reuptake into SR and extrusion by NCX. Under physiological steady-state conditions, Ca$^{2+}$ influx equals Ca$^{2+}$ efflux and there is little change in SR Ca$^{2+}$ content.

There are important differences in intracellular Ca$^{2+}$ cycling between atrial and ventricular cardiomyocytes. Compared with ventricular myocytes, SR Ca$^{2+}$ reuptake is higher in atrial myocytes, likely due to greater expression of SERCA2a and lower levels of SERCA2a-inhibitory phospholamban (PLB). Atrial myocytes exhibit higher SR Ca$^{2+}$ content and cellular Ca$^{2+}$-buffering capacity than ventricular myocytes, which is consistent with an enhanced SR Ca$^{2+}$ reuptake via SERCA2a. The stronger Ca$^{2+}$-buffering power of atrial myocytes may also result from altered Ca$^{2+}$-binding kinetics to myofilaments. Atrial NCX currents are smaller compared with those of ventricular cells. However, atrial myocytes are also smaller than ventricular myocytes, and correcting NCX current amplitude for cell size showed that atrial myocytes have larger NCX current density compared with ventricular cells.

RyR2 is the major SR Ca$^{2+}$-release channel in the heart. The sensitivity of RyR2 to cytosolic and luminal (intra-SR) Ca$^{2+}$ and thus its open probability are modulated by accessory binding proteins (e.g. calsequestrin, junctin, triadin, FK506-binding protein 12.6 kDa [FKBP12.6]) and posttranslational modifications (e.g. phosphorylation) (Figure 2A). Calsequestrin (CSQ), the major SR Ca$^{2+}$ buffer and sensor, is linked to RyR2 via junctin and triadin. Changes in the relative amounts of junctin and triadin were shown to modulate RyR2 Ca$^{2+}$ release and cause cardiac arrhythmias. Protein kinase A (PKA) and Ca$^{2+}$/calmodulin-dependent protein kinase II (CaMKII)
bind to RyR2 macromolecular complex, which enables these enzymes to dynamically phosphorylate RyR2. Conversely, RyR2-bound type-1 (PP1) and type-2A (PP2A) protein phosphatases can dephosphorylate RyR2 depending on relative kinase-phosphatase activity balance. The relative level of RyR2 phosphorylation determines RyR2 sensitivity to cytosolic Ca$^{2+}$ and thus open probability of RyR2 and the amount of SR Ca$^{2+}$ release during diastole and systole.

4. Defective SR Ca$^{2+}$ release through RyR2 channels in AF

Multiple studies have shown that abnormal SR Ca$^{2+}$ handling plays a central role in initiation and/or maintenance of chronic AF (cAF) in humans. Defective Ca$^{2+}$ handling was shown to predispose to this spontaneous SR Ca$^{2+}$ release in atrial myocytes from cAF patients (Figure 2B). SR Ca$^{2+}$ load was not increased in cAF patients, suggesting that this spontaneous SR Ca$^{2+}$ release most likely occurred because of changes in RyR2. Altered RyR2 function in cAF is accompanied by an increase in NCX expression and function, suggesting that diastolic SR Ca$^{2+}$ leak can be amplified by NCX, triggering ectopic focal discharges or facilitating microreentry circuits promoting AF maintenance.

Expression levels of RyR2 were unaltered or reduced in dogs, goats, and cAF patients, respectively. However, and perhaps more importantly, the binding levels of accessory subunits and posttranslational modification were altered in cAF, leading to increased open probability of RyR2 (Figure 3A–C). Protein levels of CSQ2 appear normal in cAF patients, whereas atrial CSQ2 levels are reduced in dogs with heart failure, likely contributing to SR dysfunction in heart failure-based AF. The level of the RyR2-stabilizing subunit FKBP12.6 (also known as calstabin2) was 50% lower in cAF patients, which could explain why RyR2 channels fail to remain closed during diastole. Consistent with this idea, FKBP12.6-deficient mice exhibit an increased vulnerability to pacing-induced AF and enhanced spontaneous SR Ca$^{2+}$ leak. It is very likely that enhanced RyR2 activity plays a role in AF pathogenesis, as mice with a gain-of-function mutation in RyR2 exhibit an increased susceptibility to pacing-induced AF. Using these knock-in mice, we demonstrated that increased SR Ca$^{2+}$ leak in atrial myocytes can promote triggered activity and atrial arrhythmias.

Changes in the phosphorylation level of RyR2 have been reported consistently in cAF (Figure 3A and B). Phosphorylation of Ser2808 (and hyperphosphorylated PLB at Ser16) in PKA-overexpressing mice exhibit an increased vulnerability to pacing-induced AF and enhanced spontaneous SR Ca$^{2+}$ leak. It is very likely that enhanced RyR2 activity plays a role in AF pathogenesis, as mice with a gain-of-function mutation in RyR2 exhibit an increased susceptibility to pacing-induced AF. Using these knock-in mice, we demonstrated that increased SR Ca$^{2+}$ leak in atrial myocytes can promote triggered activity and atrial arrhythmias.
autophosphorylation and thus increased activity of CaMKII along with higher CaMKII-dependent RyR2 phosphorylation at Ser2815,
50 clearly suggesting that the high-atrial rate is sufficient to cause these alterations. Notably, goats with atrial dilatation, but without sustained AF, also exhibit increased CaMKII activity and RyR2 hyperphosphorylation at Ser2815,
50 pointing to the possibility that structural atrial diseases may predispose to AF by producing changes in cellular Ca2+
+ signalling similar to those observed in patients being in cAF. Consistent with the critical role of enhanced CaMKII activity for AF pathophysiology, mice with a genetic gain-of-function defect in RyR2 (RyR2R176Q/+
+mice) show enhanced SR Ca2+
+ leak and increased vulnerability to rapid atrial pacing-induced AF compared with wild-type mice.51 Both genetic CaMKII inhibition or blockade of CaMKII with KN-93 prevented pacing-induced AF in these knock-in mice, clearly suggesting that inhibition of CaMKII effectively control AF inducibility in mice.51 Moreover, the absence of carbachol-induced AF in RyR2-S2814A knock-in mice, in which CaMKII phosphorylation of RyR2 was genetically inhibited, confirmed the importance of this single phosphorylation event in the pathogenesis of atrial arrhythmias.51

The molecular mechanisms of enhanced steady-state RyR2 phosphorylation at Ser2808 and Ser2814 in cAF are poorly understood. Although total protein levels and activities of PP1 and PP2A are increased in atria of cAF patients, the actual PP1 and PP2A activities within the RyR2 macromolecular complex are unknown. In addition, PP1 is regulated by inhibitor-1 and inhibitor-2.59 Inhibitor-1 levels were not altered in cAF patients, but Thr35 phosphorylation of inhibitor-1, which controls PP1 function exclusively at the SR, specifically targeting phosphorylation of PLB and RyR2 at Ser16 (PKA-site) and Ser2814 (CaMKII-site), respectively,60,61 was increased. This should lead to a strong PP1 inhibition at specific phosphorylation sites within the SR compartment, possibly contributing to enhanced RyR2 phosphorylation at Ser2814.50 The mechanism of enhanced RyR2 phosphorylation at Ser2008 in cAF is less clear. In atria of goats with sustained AF basal and cAMP-induced PKA activities were 50% lower, rendering an increase in PKA activity unlikely. As CaMKII phosphorylate Ser2808/2809,62 it could be speculated that within the SR compartment, the AF-related increase in CaMKII activity overcomes the enhanced PP1 activity, causing greater steady-state Ser2808 phosphorylation. Indeed, application of tetracaine, which decreases Ca2+
+ sensitivity of RyR2, in both quiescent and voltage-clamped (at –80 mV) human atrial myocytes in the absence of Na+
+ and Ca2+
+ in bath solution (to prevent transsarcolemmal fluxes) caused a stronger decrease in diastolic [Ca2+], in cAF patients, unmasking a larger SR Ca2+
+ leak in cAF vs. sinus-rhythm patients.41,42 Moreover, inhibition of CaMKII abolished Ca2+
+ sparking and normalized SR Ca2+
+ leak in cAF to levels seen in sinus-rhythm patients,41,42 pointing to the possibility that the AF-associated increase in SR Ca2+
+ leak is mediated exclusively by CaMKII.

In the absence of alterations in other Ca2+
+-handling proteins or atrial remodelling, increased open probability of RyR2 alone (Figure 3C) is probably not sufficient to trigger spontaneous Ca2+
+ waves causing DADs/triggered activity.51,63 It is possible that diastolic SR Ca2+
+ leak via RyR2 can only be sustained if a certain threshold of SR Ca2+
+ load can be maintained to ensure sufficient RyR2 sensitization to luminal
(intra-SR) Ca\textsuperscript{2+}. SR Ca\textsuperscript{2+} load not only determines the size of the Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release by the law of mass action, but also sensitizes RyR2 to luminal Ca\textsuperscript{2+} by an allosteric interaction that changes RyR2 gating properties. In dogs with tachycardio-myopathic heart failure, the luminal SR Ca\textsuperscript{2+} concentration that causes half-maximal RyR2 acti-

vation is about 100 times lower than that in control animals, and similar increases in sensitivity to luminal Ca\textsuperscript{2+} were reported for some RyR2 mutations related to CPVT. These studies clearly demonstrate that disease-related changes in the ability of RyR2 to sense luminal Ca\textsuperscript{2+} may promote diastolic SR Ca\textsuperscript{2+} leak in the presence of both normal and reduced SR Ca\textsuperscript{2+} load. However, it remains to be determined experimentally whether the sensitivity of RyR2 to luminal Ca\textsuperscript{2+} is changed in AF. Thus, the specific consequences of changes in SR Ca\textsuperscript{2+} load are not as clear, because SR Ca\textsuperscript{2+} load modulates sensitivity of RyR2 to luminal Ca\textsuperscript{2+} and this may have important implications for subsequent phosphorylation-dependent regulation of RyR2 sensitivity to cytosolic Ca\textsuperscript{2+}. Although SR Ca\textsuperscript{2+} load appears normal in cAF (Figure 4A and B), which may play a permissive role for RyR2 dys-

function, the mechanisms that help to maintain sufficient SR Ca\textsuperscript{2+} content are poorly understood. In cAF patients, PLB is hyperphosphorylated at both Ser16 (PKA-site) and Thr17 (CaMKII-site), respect-

ively, which may prevent SR Ca\textsuperscript{2+} depletion during AF, potentially contribut-
ing to preserved SR Ca\textsuperscript{2+} content. Again, elevated PLB phosphorylation occurs in the face of globally increased activity levels of PP1 and PP2A, which highlights the importance of local differences in protein phosphatase activity and/or targeting within discrete microdomains in atrial myocytes. In addition, it has been demonstrated that the expression levels of sarcolipin (SLN), an SERCA2a inhibitor that like PLB loses its SERCA2a-inhibitory properties when phosphorylated by CaMKII at Thr5, is decreased in cAF patients. Reduced SLN binding to SERCA2a together with altered PLB regulation could theoretically enhance SR Ca\textsuperscript{2+} reuptake, offsetting the Ca\textsuperscript{2+} loss due to increased SR Ca\textsuperscript{2+} leak.

In addition, amplitude of \( L_{Ca,L} \) is ~50–70% lower in cAF patients, and reduced SR Ca\textsuperscript{2+} release due to the decreased trigger \( L_{Ca,L} \) might be the major cause for the ~50% smaller Ca\textsuperscript{2+}-transient amplitude in cAF patients (Figure 5) because SR Ca\textsuperscript{2+} content is unchanged. \( L_{Ca,L} \) is subject to Ca\textsuperscript{2+}-dependent inactivation, and SR Ca\textsuperscript{2+} release contributes importantly to Ca\textsuperscript{2+}-dependent \( L_{Ca,L} \) inactivation creating a negative feedback on Ca\textsuperscript{2+} influx. In fact, the smaller Ca\textsuperscript{2+}-transient amplitude in cAF patients allows larger time-dependent Ca\textsuperscript{2+} influx and the integrated Ca\textsuperscript{2+} influx through \( L_{Ca,L} \) is only ~25% lower in cAF vs. sinus-rhythm patients (N. Voigt et al., unpublished observations). In addition, as under steady-state conditions, Ca\textsuperscript{2+} influx equals Ca\textsuperscript{2+} efflux with little change in SR Ca\textsuperscript{2+} content, the lower \( L_{Ca,L} \) in cAF will trigger smaller release from the SR (Figure 5) and accordingly less Ca\textsuperscript{2+} is pumped out of the cell by NCX with each beat, which may possibly explain why there is little change in SR-Ca\textsuperscript{2+} load in cAF patients. Thus, in cAF patients, normal SR Ca\textsuperscript{2+} load might be maintained through multiple mechanisms.

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**Figure 4** Increased NCX function in patients with cAF. (A) Representative examples of caffeine-evoked Ca\textsuperscript{2+} transients (CaT) in voltage-clamped (at ~80 mV) atrial myocytes from patients in sinus rhythm (SR) or cAF. (B) Bar graphs showing no significant differences in sarcoplasmic reticulum Ca\textsuperscript{2+} content evidenced by caffeine-evoked CaT amplitude and integrated Na\textsuperscript{+}/Ca\textsuperscript{2+}-exchange current (\( I_{NCX} \)). The non-significantly smaller caffeine-evoked CaT amplitude, but unaltered integrated \( I_{NCX} \) current in cAF points to a potentially higher Ca\textsuperscript{2+}-buffering capacity in cAF vs. SR patients, but this requires further validation in subsequent work (see text for further details). (C) Western blots showing increased NCX1 expression levels in cAF. (D) \( I_{NCX} \) evoked by caffeine application reveals a greater \( I_{NCX} \) for a given \([Ca^{2+}]_{o}\) in AF vs. SR patients. (E) Bar graphs showing an increased slope of the \( I_{NCX} \) and faster decay of caffeine-evoked CaT in cAF as indices of increased functional NCX. (F) Trend toward increased \( I_{NCX} \) peak current in cAF. Numbers within parentheses indicate myocytes/patients. Reproduced with permission from Voigt et al.45
Interestingly, dogs with experimental heart failure show increased SR Ca\(^{2+}\) load, along with enhanced CaMKII phosphorylation of PLB and increased frequency of DADs in atrial cells,\(^7^2\) suggesting that DADs and triggered activity might occur particularly in heart failure-induced AF.

### 5. Upregulation of NCX may amplify the consequences of increased diastolic SR Ca\(^{2+}\) leak in AF

It is believed that NCX can contribute to arrhythmogenesis in AF by its ability to generate a transient inward current (\(I_{\text{ti}}\)) following forward-mode activation triggered by SR Ca\(^{2+}\) release.\(^2^0\) By removing excess cytosolic Ca\(^{2+}\) during diastole, NCX promotes Na\(^{+}\) entry which could initiate abnormal APs and triggered activity, thereby contributing to atrial arrhythmogenesis. Moreover, it is believed that \(I_{\text{ti}}\) in atrial myocytes is largely mediated by NCX without significant contributions by Ca\(^{2+}\)-dependent chloride or non-selective cation currents.\(^2^7\) The expression levels of NCX1 are upregulated in patients and sheep with cAF, consistent with a potential role for NCX in the generation of DADs.\(^4^1,4^2,4^3\)

The molecular mechanisms underlying increased NCX activity in AF is unknown. NCX is organized in a macromolecular complex that contains kinases (protein kinases A and C), phosphatases (PP1 and PP2A), and multiple regulatory proteins (ankyrin-B, \(\beta\)-spectrin, actin) that are proposed to exert tonic NCX modulation.\(^7^2\) Thus, regulatory proteins and posttranslational modifications could also contribute to enhanced NCX activity, but the impact of impaired regulation on altered NCX function requires further investigation. The larger amount of NCX1 protein in AF is probably due to the increase in NCX1 mRNA levels, which are reversible after restoration of sinus rhythm in cAF patients.\(^7^3\) This points to the possibility that increased NCX1 expression is the consequence of AF itself, contributing to maintenance rather than induction of AF. Regardless of the underlying molecular mechanism, the enhanced function of NCX in response to a given SR Ca\(^{2+}\) release (Figure 4D and F) in combination with the increased diastolic SR Ca\(^{2+}\) leak\(^4^1,4^2\) may cause DADs and triggered activity that may contribute to AF maintenance.

### 6. Potential relevance of spontaneous RyR2 Ca\(^{2+}\) release for AF pathophysiology

It is well recognized that AF is a progressive condition, with AF-induced atrial remodelling increasing the susceptibility to and stability of AF.\(^7^4\) Multiple factors underlie atrial remodelling\(^7^4\) and there is now emerging evidence that altered atrial subcellular Ca\(^{2+}\) signalling may cause DADs/ triggered activity that may promote the induction and/or maintenance of AF.\(^7^5\) SR Ca\(^{2+}\) leak in AF appears to result from increased CaMKII activity with subsequent RyR2 hyperphosphorylation (Figure 3B).\(^8^1,4^2,5^1\) Spontaneous Ca\(^{2+}\) waves in ventricular myocytes from failing rabbit hearts depend on CaMKII,\(^7^5\) suggesting that CaMKII-mediated SR Ca\(^{2+}\) leak in AF might cause potentially arrhythmogenic spontaneous Ca\(^{2+}\) waves. It is generally assumed that SR Ca\(^{2+}\) leak and waves of spontaneous Ca\(^{2+}\) release result from similar mechanisms, but sustained SR Ca\(^{2+}\) leak could produce a background \(I_{\text{NCX}}\) rather than a triggering current. Furthermore, spontaneous Ca\(^{2+}\) waves are slow and dysynchronous and it is therefore unlikely that under resting conditions diastolic Ca\(^{2+}\) waves will cause DADs that are sufficiently large for generating triggered APs.\(^7^6\) The latter can only occur if the physiological safety margin decreases,\(^1^9\) for instance, due to remodelling-induced changes in [Ca\(^{2+}\)]\(_i\)–membrane voltage coupling gain. Indeed, \(I_{\text{NCX}}\) increases linearly with the rise of [Ca\(^{2+}\)]\(_i\), but a given [Ca\(^{2+}\)]\(_i\) generates a larger \(I_{\text{NCX}}\) in cAF (Figure 4D and E).\(^4^4,4^5\) Suggesting an increased coupling gain between [Ca\(^{2+}\)]\(_i\) and \(I_{\text{NCX}}\) in cAF. However, the degree of subsequent membrane depolarization is nonlinear, because the stability of the resting membrane potential (RMP) depends on multiple factors including activity of the inward-rectifier \(I_{\text{K1}}\) and possibly other background currents.\(^7^0\) As the amplitude of inward-rectifier currents, which are the major determinants of RMP, is larger and is associated with a slightly more negative RMP in cAF patients,\(^7^7–8^2\) cellular DADs and triggered AP can occur only if there is an AF-associated change in the diastolic [Ca\(^{2+}\)]\(_i\)–membrane voltage coupling gain.\(^1^9,8^3\)

It is very likely that altered RyR2 and NCX functions contribute to atrial arrhythmogenesis in AF by providing an arrhythmogenic substrate, especially in vivo whereby the high-atrial rate (5–8 Hz) and the neurohumoral influences should amplify the consequences of altered cellular Ca\(^{2+}\) signalling potentially serving as triggers (Figure 6). However, there are still important gaps in our knowledge about impaired atrial Ca\(^{2+}\) handling. For instance, persistence of diastolic SR Ca\(^{2+}\) leak requires maintained SR Ca\(^{2+}\) load.\(^9^4\) Although recent results in AF patients point to preserved global SR Ca\(^{2+}\) load and enhanced functional NCX,\(^4^1,4^3\) it remains to be determined whether increased SR Ca\(^{2+}\) leak and sufficient SR Ca\(^{2+}\) load occur at the sub-sarclemmal SR compartment. Although SR Ca\(^{2+}\) leak is higher in cAF vs. sinus-rhythm patients,\(^9^1,9^2\) the quantitative relation between SR Ca\(^{2+}\) leak, probability of occurrence of diastolic Ca\(^{2+}\) waves, and amplitude of NCX is currently unknown. Nevertheless, the size of the NCX current associated with
spontaneous SR Ca²⁺ release appears sufficient to depolarize the membrane to the threshold needed for triggering an AP because preliminary results show that incidence of spontaneous (non-stimulated) Ca²⁺-release events accompanied by corresponding inward $I_{NCX}$ currents is higher in cAF patients, and cellular DADs/triggered APs occur more often in patients with cAF than those in sinus rhythm. This makes an increase in coupling gain between alterations in diastolic [Ca²⁺]i and changes in membrane voltage in AF patient very likely, but this remains to be determined.

In addition to DADs and triggered activity, spontaneous Ca²⁺ waves may also cause subcellular Ca²⁺ alternans and sudden repolarization changes that may increase dispersion of refactoriness,86 promoting reentry, and arrhythmia maintenance (Figure 6). Finally, although Ca²⁺-dependent sources are suggested to contribute to maintenance of clinical AF,97 direct experimental evidence of the causal relationship between Ca²⁺-related cellular proarrhythmic events and focal sources and/or continuous conduction in fibrillating human atria is still lacking. Thus, additional work will be needed to definitely prove the clinical impact of altered atrial Ca²⁺ signalling for AF promotion and maintenance.

7. Potential therapeutic opportunities to target abnormal RyR2 function in AF

Traditionally, AF has been treated with drugs that block voltage-gated ion channels. However, current drug therapy is also associated with serious proarrhythmic effects, which strongly limits its clinical applicability.2 There is hope that a better understanding of molecular pathways involved in atrial arrhythmogenesis will lead to the development of safer and more effective therapeutic approaches for AF treatment.2,14

New therapeutic modalities may include compounds that inhibit SR Ca²⁺ leak by normalizing the function of the RyR2 macromolecular complex.21 The 1,4-benzothiazepine analogue JTV519 (K201) prevents AF in a canine model of sterile pericarditis88 and reduces the RyR2-mediated SR Ca²⁺ leak in mice by reversing disease-associated reduction in FKBP12.6 binding to RyR2.35,89,90 In addition, JTV519 reduces the incidence of spontaneous SR Ca²⁺ release and DADs that arise as a consequence of SR Ca²⁺ leak in mouse ventricular myocytes.22 It also reduces firing rates in rabbit pulmonary vein cardiomyocytes, decreases amplitude of DADs, prolongs AP duration, and decreases incidence of pacing-induced AF.91 In addition to RyR2, JTV519 also affects several atrial92 and pulmonary vein ion channels.91 JTV519 appears a suitable lead structure for development of drugs that specifically target RyR2 function and the RyR2/FKBP12.6 interaction. A more specific JTV519 analogue (S107)93 is currently under investigation, but efficacy and safety of S107 remains to be determined in experimental AF paradigms and AF patients.

RyR2 blocking agents may also be of potential therapeutic value. For example, the local anaesthetic drug tetracaine completely suppresses Ca²⁺ sparks in atrial myocytes from patients in cAF.40,41 Flecainide, a class 1C anti-arrhythmic drug blocking voltage-gated Na⁺ channels and K⁺ channels known to promote AF maintenance,94 effectively inhibits arrhythmias due to SR Ca²⁺ leak in mouse ventricular...
myocytes. 95 It also inhibits intracellular Ca$^{2+}$ waves, probably due to a combined effect on RyR2 gating and voltage-gated Na$^+$ channels. 96,97 Interestingly, in contrast to flecainide, tetracaine does not inhibit Ca$^{2+}$-wave propagation and this likely results from the different mechanism of action of tetracaine on RyR2 gating. 96 Tetracaine stabilizes RyR2 channels in their closed state reducing RyR2 open probability and increasing SR Ca$^{2+}$ content, whereas flecainide is an open-channel blocker that decreases RyR2 mean-open time, thereby reducing Ca$^{2+}$-spark mass, although Ca$^{2+}$-spark frequency was increased, which could explain why flecainide does not increase SR Ca$^{2+}$ content. The smaller Ca$^{2+}$-spark mass could prevent Ca$^{2+}$ waves, because it makes salutary Ca$^{2+}$-wave propagation which may induce DADs less likely. Although flecainide is ideally suited to suppress arrhythmogenic Ca$^{2+}$ waves without causing compensatory increases of SR Ca$^{2+}$ content, 96 the primary action of flecainide in vivo is to inhibit Na$^+$ channels which may cause malignant ventricular arrhythmias, especially if applied chronically in patients with severe coronary artery disease. Analogue substances causing open-channel block of RyR2 without effects on Na$^+$ channels may have anti-AF efficacy without collateral effects at the ventricular level.

In addition to direct inhibition of RyR2, SR Ca$^{2+}$ leak may be reduced by suppressing the activity of CaMKII in the atrium. Local targeting of kinase and phosphatase functions (e.g. targeting of inhibitor-1 of PP1) is a possible, but yet unproven, therapeutic strategy. Nevertheless, we demonstrated that pharmacological inhibition of CaMKII could inhibit the induction of AF in mice with mutant RyR2 channels by reducing SR Ca$^{2+}$ leak. 51 In addition, inhibition of calmodulin should prevent activation of CaMKII and could rescue the down-regulation of Ca$^{2+}$ sparks without effects on SR Ca$^{2+}$ content. 98 However, general targeting of CaMKII might exert adverse effects on memory and fertility 99 and can cause negative-inotropic effects, 17,100 and targeting the mechanisms of increased cardiac CaMKII activity could be one alternative approach. Oxidative stress-induced afterdepolarizations are linked to CaMKII signaling 94 and it is known that abnormal CaMKII activity might also result from oxidative stress and increased angiotensin-II levels that cause CaMKII oxidation at Met281/282, leading to sustained CaMKII activation. 101 Although it is unknown whether atrial CaMKII is hyperoxidized in AF, oxidative stress and inflammation are the hallmarks of AF. 102,103 Therefore, it is possible that at least part of the efficacy of current anti-oxidative/anti-inflammatory therapeutic anti-AF options using statins, ACE-inhibitors, and AT1-receptor blockers result from inhibition of CaMKII activation. Thus, use of statins and ACE-inhibitors/AT1-receptor blockers to suppress abnormal CaMKII activation and subsequent RyR2 hyperphosphorylation in combination with open-channel RyR2 blockers and/or stabilizer of RyR2-FKBP12.6 binding may prove to be a useful principle in future anti-AF drug therapy targeting defective RyR2 function.

8. Conclusions and perspectives

There is emerging evidence that increased diastolic SR Ca$^{2+}$ leak along with enhanced NCX function may cause DADs and triggered activity that contribute to AF maintenance. Recent work in genetically modified mice 51,53 have provided important insights into the causal relationships between molecular alterations of RyR2 and AF susceptibility, clearly validating the importance of these specific Ca$^{2+}$-handling alterations for AF pathophysiology. However, it remains to be established whether these cellular Ca$^{2+}$-related proarrhythmic events contribute to atrial arrhythmogenic foci in AF patients in vivo. Nevertheless, the development of new drugs specifically targeting arrhythmogenic diastolic SR Ca$^{2+}$ leak might offer unique therapeutic opportunities to reduce atrial arrhythmogenesis by normalizing SR Ca$^{2+}$ handling.

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