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TOPICAL REVIEW

Calcium-mediated cellular triggered activity in atrial fibrillation

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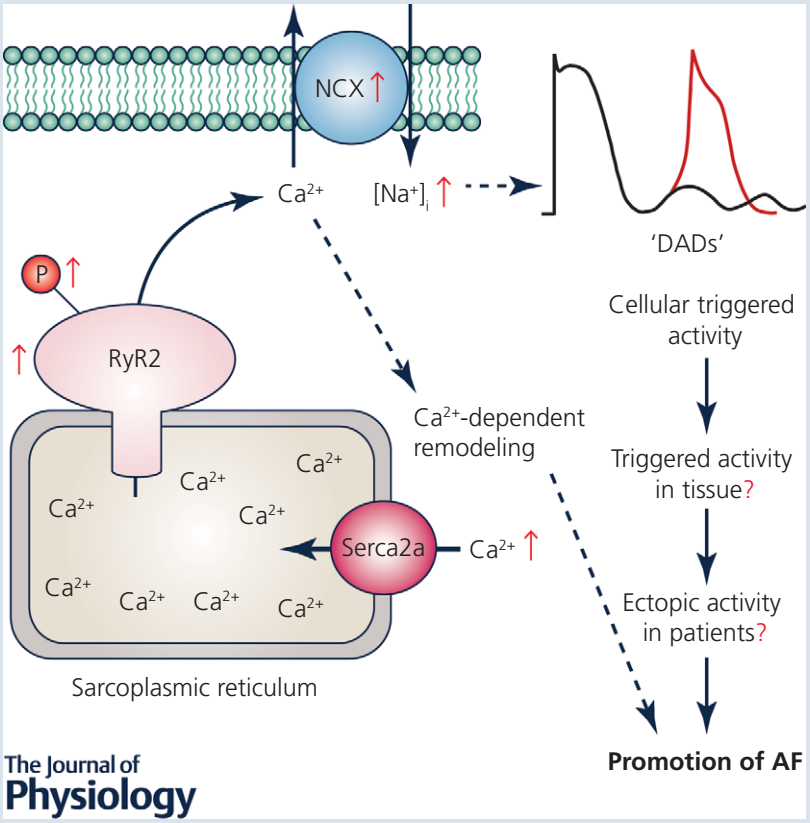

Abstract Although atrial fibrillation (AF) is the most commonly encountered cardiac arrhythmia, the basic mechanisms underlying this disorder remain incompletely understood. During the past

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decade or so, it has become clear that alterations in intracellular Ca^{2+} handling may play a role in the pathogenesis of AF. Studies in small and large animal models, as well as atrial samples from patients with different forms of AF, have implicated ryanodine receptor type 2 (RyR2) dysfunction and enhanced spontaneous Ca^{2+} release events from the sarcoplasmic reticulum (SR) as a potential cause of proarrhythmic cellular ectopic (triggered) activity in AF. The molecular mechanisms leading to RyR2 dysfunction and SR Ca^{2+} leak depend on the clinical stage of AF or specific animal model studied. This review focuses on the mechanisms and role of calcium-mediated cellular triggered activity in AF, and addresses some of the current controversies in the field.

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Abstract figure legend Schematic diagram showing molecular mechanisms underlying abnormal SR Ca^{2+} release associated with ectopic (triggered) activity in atrial fibrillation. Arrows indicate the direction of change. CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; NCX, Na^+/Ca^{2+}-exchanger; PKA, protein kinase A; PP1, protein phosphatase 1; RyR2, ryanodine receptor type-2; SERCA2a, sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a.

Abbreviations AF, atrial fibrillation; cAF, long-standing persistent (chronic) AF; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CPVT, catecholaminergic polymorphic ventricular tachycardia; DAD, delayed afterdepolarization; EAD, early afterdepolarization; JPH2, junctophilin 2; LTCC, L-type Ca^{2+} channel; NCX, Na^+/Ca^{2+}-exchanger; pAF, paroxysmal AF; PP1, protein phosphatase 1; PKA, protein kinase A; RyR2, ryanodine receptor type 2; SERCA2a, sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a; SR, sarcoplasmic reticulum.

Atrial fibrillation (AF) is the most common type of cardiac arrhythmia with a rising incidence and prevalence in the developed world (Andrade *et al.* 2014). AF is a progressive disease that can be classified into paroxysmal AF (pAF), which converts spontaneously to sinus rhythm within 7 days (usually less than 48 h), persistent AF, which lasts for more than 7 days, long-standing persistent (chronic) AF (cAF), which lasts for over 1 year, and permanent forms, for which no further attempts are made to restore sinus rhythm (Kirchhof *et al.* 2016). Although our knowledge about the pathophysiology of AF has expanded greatly during the last 20 years, particularly about the cellular and molecular basis of AF-promoting atrial remodelling, the precise mechanisms leading to AF have remained elusive (Heijman *et al.* 2014*a*).

Independent of the underlying cause, which may be clinically diverse, it is believed that abnormal impulse formation (focal ectopic activity) and reentry are the two major determinants of AF initiation and maintenance, although the precise clinical correlates of these fundamental mechanisms are not uniformly validated and are somewhat controversial (Schotten *et al.* 2016). In the clinical setting atrial ectopy may occur as a result of autonomic nervous system imbalance/altered innervation, acute ischaemia, wall stress (mechanosensing), and other macroscopic determinants of arrhythmogenesis (Andrade *et al.* 2014). A growing body of evidence suggests that abnormal intracellular Ca^{2+} handling may play a role in both the initiation of AF episodes and cellular remodelling processes that drive AF progression to more persistent forms. This review article will provide a critical examination of the experimental evidence that cellular ryanodine receptor type 2 (RyR2)-mediated triggered activity may contribute to the pathophysiology of AF.

Basic mechanisms of triggered activity in AF

Triggered activity can be caused at the cellular level by early or delayed afterdepolarizations (EADs and DADs, respectively). EADs involve a secondary depolarization before final repolarization of the primary action potential as a result of reopening of L-type Ca^{2+} channels and in rare cases by reactivation of fast Na^+ currents. On the other hand, DADs occur after completion of repolarization of the primary action potential as a result of spontaneous Ca^{2+} release events from the sarcoplasmic reticulum (SR). These SR Ca^{2+} release events through ryanodine receptor/intracellular Ca^{2+} release channels (RyR2) activate the Na^+/Ca^{2+}-exchanger (NCX), which brings three Na^+ ions in for each Ca^{2+} ion pumped out of the cell, producing a depolarizing transient inward current that can cause a triggered action potential leading to focal ectopic firing (Voigt *et al.* 2012; Heijman *et al.* 2014*a*). Repetitive focal ectopic activity may sustain AF even in the absence of an AF-maintaining substrate. On the other hand, focal ectopic firing can also initiate a reentrant circuit that can maintain AF. Reentry is considered the main mechanism for AF maintenance, and can occur around anatomical obstacles

or can be functional in nature (Heijman *et al.*, 2012, 2016).

Role of calcium in triggered activity in AF

Contraction of the atria is initiated by electrical depolarization of the myocytes, which initiates opening of voltage-dependent L-type Ca^{2+} channels. The influx of Ca^{2+} into the cytosol then triggers an amplified release of Ca^{2+} from the SR via RyR2 Ca^{2+} release channels (McCauley & Wehrens, 2011). The elevated cytosolic Ca^{2+} levels induce sarcomere shortening and atrial contraction. Relaxation occurs when cytosolic Ca^{2+} concentrations are brought back to diastolic levels by pumping Ca^{2+} back into the SR via the SR Ca^{2+}-ATPase 2a (SERCA2a), or by extruding Ca^{2+} through NCX on the plasma membrane. The amplitude of Ca^{2+}-induced Ca^{2+} release depends on proper alignment of L-type Ca^{2+} channel (LTCC) on the plasma membrane and RyR2 on the SR by a structural protein known as junctophilin-2 (JPH2) (Landstrom *et al.* 2014). In addition, JPH2 binding to RyR2 prevents abnormal diastolic SR Ca^{2+} leak (Beavers *et al.* 2013). The open probability of RyR2 is also regulated by phosphorylation by protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and dephosphorylation by protein phosphatase 1 (PP1) in atrial myocytes.

Abnormal SR Ca^{2+} leak has emerged as a potential contributor to cellular triggered activity in right atrial cardiomyocytes of both pAF and cAF patients (Voigt *et al.* 2012, 2014; Heijman *et al.* 2014*b*; Abstract figure). In patients with pAF, an increased frequency of spontaneous SR Ca^{2+} release events was observed even though sinus rhythm was present at the time of tissue collection excluding rapid atrial rate-induced remodelling as an underlying cause (Beavers *et al.* 2013; Voigt *et al.* 2014). The underlying molecular substrate was found to involve enhanced SR Ca^{2+} loading resulting from increased SERCA2a activity (Voigt *et al.* 2014). In addition, increased protein expression and open probability of RyR2 channels contributed to the increased frequency of spontaneous SR Ca^{2+} release events and related DADs in pAF (Voigt *et al.* 2014). Increased expression of RyR2 has been attributed to reduced levels of the inhibitory microRNA-106b-25 cluster in pAF patients (Chiang *et al.* 2014*a*). Moreover, a reduction in the ratio between RyR2-stabilizing protein JPH2 and RyR2 may contribute to an increased RyR2 channel open probability, although the precise mechanisms of SR Ca^{2+} leak in pAF patients remain incompletely understood (Beavers *et al.* 2013). Further research is needed to elucidate the precise molecular mechanisms underlying the dysfunction of RyR2 and the related propensity to cellular triggered activity in pAF patients, and to validate whether these potentially proarrhythmic cellular events indeed contribute to the initiation and recurrence of AF episodes in pAF patients *in vivo*.

In patients with cAF, Ca^{2+}-handling abnormalities and DAD-mediated cellular triggered activity have also been demonstrated in right atrial cardiomyocytes from patients undergoing cardiac surgery (Hove-Madsen *et al.* 2004; Liang *et al.* 2009; Neef *et al.* 2010; Voigt *et al.* 2012). At the molecular level, enhanced phosphorylation or oxidation of RyR2 along with an increased RyR2 open probability have been proposed to cause Ca^{2+}-handling abnormalities in cAF patients (Vest *et al.* 2005; Voigt *et al.* 2012; Fischer *et al.* 2015; Xie *et al.* 2015). Studies in a mouse model of AF revealed a potential role for CaMKII in the development of triggered activity in AF (Chelu *et al.* 2009). The critical role of dysfunctional RyR2 for cellular triggered activity was confirmed in several other small and large animal models (Neef *et al.* 2010; Nishida *et al.* 2011; Li *et al.* 2012; Shan *et al.* 2012; Kettlewell *et al.* 2013; Purohit *et al.* 2013; Faggioni *et al.* 2014; Guo *et al.* 2014; Macquaide *et al.* 2015; Zhang *et al.* 2015), as well as human atrial cardiomyocytes isolated from cAF patients (Neef *et al.* 2010; Voigt *et al.* 2012). In addition, RyR2 may be more leaky due to increased PKA-mediated phosphorylation although there is less evidence supporting this notion (Vest *et al.* 2005, Voigt *et al.* 2012; Li *et al.* 2012). Finally, reduced activity of protein phosphatases that dephosphorylate RyR2 can also contribute to enhanced phosphorylation of RyR2 and increased SR Ca^{2+} leak (Chiang *et al.* 2014*b*, 2015). Thus, extensive evidence from animal models and patients support a potential role of SR Ca^{2+} leak and associated cellular DAD-mediated triggered activity in patients with persistent AF.

Finally, there is emerging evidence that SR Ca^{2+} leak may directly promote the progression of AF to more persistent forms. In a mouse model of spontaneous progressive AF, inhibition of RyR2 phosphorylation (and thus, enhanced RyR2-mediated SR Ca^{2+} leak) was shown to prevent the development of a substrate for AF maintenance despite the presence of atrial triggered activity (Li *et al.* 2014). These findings suggest that RyR2-mediated SR Ca^{2+} leak drives Ca^{2+}-dependent remodelling pathways (such as the calcineurin/NFAT pathway) that contribute to the formation of AF-promoting atrial remodelling and an arrhythmogenic substrate for AF persistence. The role of Ca^{2+}-dependent remodelling in AF progression has been discussed in greater detail in another recent review and still needs to be evaluated in large animal models and patients (Heijman *et al.* 2014*a*).

Controversies regarding the role of SR Ca^{2+} leak in AF pathophysiology

An ideal way to test the hypothesis that SR Ca^{2+} leak though RyR2 is responsible for triggered activity in

patients with AF would be to conduct a clinical trial in which highly specific pharmacological RyR2 channel modulators would be tested. To the best of our knowledge, such clinical trials have not been conducted to date. Several groups have developed RyR2 channel modulators – some more RyR2-specific than others – that have been tested in animal models and tissue samples from patients with heart disease (Nakaya *et al.* 2000; Kumagai *et al.* 2003; Wehrens *et al.* 2004; Loughrey *et al.* 2007; Toischer *et al.* 2010; Sadrpour *et al.* 2015). For example, 1,4-benzothiazepine derivative K201 (JT519) was shown to terminate atrial flutter and AF in canine models of sterile pericarditis (Kumagai *et al.* 2003; Sadrpour *et al.* 2015). This particular compound has many off-target effects in addition to RyR2 inhibition. Further studies are needed to test whether the aforementioned and newer generation RyR2 inhibitors can actually suppress triggered activity in patients with AF.

There is strong clinical evidence that genetic gain-of-function defects in RyR2 – in patients with 'catecholaminergic polymorphic ventricular tachycardia' (CPVT) and otherwise normal hearts – develop cardiac arrhythmias including AF (Paavola *et al.* 2007). Although initially neglected, it is now established that individuals with CPVT have a higher propensity to both ventricular and atrial arrhythmias including AF. Most important, DADs were recorded in a patient with CPVT, which demonstrated for the first time that genetic RyR2 dysfunction might directly cause atrial triggered activity in the clinical setting (Paavola *et al.* 2007). Future studies are needed to elucidate whether gain-of-function defects of RyR2 in patients with non-genetic forms of AF also contribute to DADs and ectopic activity.

Because of better clinical availability, most of the work dealing with RyR2 dysfunction was performed in right atrial cardiomyocytes from patients with AF (Hove-Madsen *et al.* 2004; Vest *et al.* 2005; Neef *et al.* 2010; Voigt *et al.* 2012; Beavers *et al.* 2013; Voigt *et al.* 2014). Although it has been a widely accepted notion that the right atrium is not a major source for AF induction and maintenance, this assumption may be incorrect. Right atrium-located AF drivers/rotors are detectable in up to 30–50% of patients with AF and in *ex vivo* perfused human hearts and in some patients only ablation of such right atrium-located drivers stops the arrhythmia (Hocini *et al.* 2010; Hasebe *et al.* 2016; Li *et al.* 2016; Spitzer *et al.* 2017). This is solid evidence that the right atrium can be a relevant source for clinical AF and accordingly studies performed in human right atrial tissue clearly provide valuable insights into the molecular basis of AF. In addition, RyR2-mediated SR Ca^{2+} leak was recently demonstrated in left atrial cardiomyocytes from AF patients (Fischer *et al.* 2015), making it likely that the RyR2 abnormalities detected in right atrial cardiomyocytes from patients with AF are not restricted to the right atrium only (Hove-Madsen *et al.* 2004; Vest *et al.* 2005; Neef *et al.* 2010; Voigt *et al.* 2012; Beavers *et al.* 2013; Voigt *et al.* 2014). Additional work in human left atrial tissue is clearly needed to further validate the role of RyR2 abnormalities for AF pathophysiology.

A few recent studies of human tissue samples and animal models have suggested that silencing of intracellular Ca^{2+} handling rather than increased SR Ca^{2+} leak contribute to AF pathogenesis (Greiser *et al.* 2014). Greiser *et al.* (2014) showed that there was no increase in SR Ca^{2+} leak despite PKA hyperphosphorylation of serine 2808 on RyR2 in rabbits with atrial tachycardia remodelling but otherwise normal atria. In their model, 5 days of atrial tachypacing at 10 Hz led to a severe downregulation of RyR2 protein levels, which according to computational modelling might offset the effects of RyR2 hyperphosphorylation on SR Ca^{2+} leak. However, such a downregulation of RyR2 has not been reported in AF patients, suggesting that this model has limited clinical relevance or represents a disease stage not previously characterized in patients or healthy individuals. In addition, the reduction in intracellular $[Na^+]$ observed in this rabbit model and dogs with atrial tachycardia (Akar *et al.* 2003) is inconsistent with findings in human atrial cardiomyocytes from cAF patients, in which a significantly (3-fold) elevated intracellular $[Na^+]$ has been reported (Hammer *et al.* 2016), although this finding needs further independent validation. Finally, rabbits with chronic myocardial infarction-induced atrial remodelling and dogs with congestive heart failure show DAD-mediated triggered activity and AF (Yeh *et al.* 2008; Kettlewell *et al.* 2013), which is in line with the phenotype of patients with AF outlined above and the emerging evidence that atrial cardiomyopathy is a critical determinant of the increased propensity to pAF and more persistent forms of AF (Goette *et al.* 2017).

Some authors have reported reduced, rather than enhanced, spontaneous arrhythmic activity in multicellular atrial trabeculae from patients with cAF compared with patients with sinus rhythm (Sossalla *et al.* 2010; Christ *et al.* 2014). However, the study of Sossalla *et al.* (2010) was not specifically designed to study DAD-mediated triggered activity and one possible explanation for the opposite results of Christ *et al.* (2014) is that they incubated the trabeculae with phenoxybenzamine, a non-selective, irreversible α-adrenoceptor blocker which inhibits calmodulin and L-type Ca^{2+} channels, among other things (Gengo *et al.* 1984; Cimino & Weiss, 1988). Irreversible inhibition of calmodulin could impact intracellular Ca^{2+} release due to inhibition of various channels, including L-type Ca^{2+} channels, Na^+ channels and RyR2. Therefore, the fact that abnormal aftercontractions (as used as an index of arrhythmias) were not more prevalent in atrial trabeculae from cAF patients as compared to sinus

rhythm patients does not disprove the importance of DAD-mediated triggered activity (Christ *et al.* 2014). If anything, it is very likely that these experiments rather proved that calmodulin inhibition is a more effective anti-arrhythmic strategy in multicellular preparations from cAF patients compared to patients in sinus rhythm. Moreover, contraction of multicellular atrial preparations is probably not a suitable readout for proarrhythmic events, particularly when atrial contraction itself is strongly impaired as commonly observed in AF patients (Schotten *et al.* 2001; Wettwer *et al.* 2004).

In their study, Christ *et al.* (2014) did not observe an increased rate of spontaneous SR Ca^{2+} release events between the regular L-type Ca^{2+}-current ($I_{Ca,L}$)-stimulated Ca^{2+} transients (CaTs), even under conditions of increased SR Ca^{2+} load with β-adrenoceptor activation, an observation which is in line with the results of Voigt *et al.* (2012) and Beavers *et al.* (2013). In order to increase the susceptibility to spontaneous CaT generation, Voigt *et al.* (2012) used 5 mM instead of 2 mM extracellular Ca^{2+} (which increases SR Ca^{2+} load) and clamped the cardiomyocytes at −80 mV (mimicking diastolic conditions) after SR Ca^{2+} loading with regular $I_{Ca,L}$ stimulation. These conditions – which simulate the situation of a pause after a run of atrial tachycardia – unmasked the higher propensity to spontaneous SR Ca^{2+} release events in atrial cardiomyocytes of cAF patients compared to controls. Moreover these findings were also validated in the current-clamp configuration (with physiological extracellular Ca^{2+}) where DADs more frequently occurred in atrial cardiomyocytes from cAF patients compared to controls. Clearly independent verification in additional cohorts of AF patients using similar patch clamp protocols is needed to further validate the presence and role of cellular triggered activity in human AF.

Conclusions

There is a growing body of evidence that abnormal RyR2 activity and enhanced SR Ca^{2+} leak may cause DADs and cellular triggered activity in animal models of and patients with AF. The molecular mechanisms underlying SR Ca^{2+} leak, in particular RyR2 dysfunction, appear to depend on the clinical stage of AF or the specific animal model studied. Further assessment of single RyR2 channel properties, comprehensive mapping of post-translational modifications of RyR2, recording of cellular DADs and evaluation of multicellular preparations from patients with different forms of AF are needed to gain a better understanding of the molecular basis of triggered activity in AF and to validate the role and importance of focal ectopic firing for atrial arrhythmogenesis. Ultimately, the only way to test the contribution of dysfunctional RyR2 channels to macroscopic AF mechanisms in suitable cohorts of AF patients is to employ highly selective RyR2 inhibitors, which are being developed by several groups but to the best of our knowledge have not been tested yet in patients with AF. Work in perfused intact human atria as successfully employed in a very recent study could be the next intermediate step in the translation of findings obtained in a cellular context to the clinical setting (Li *et al.* 2016).

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Additional information

Competing interests

X.H.T.W. is a founding partner of Elex Biotech, a start-up company that develops drug molecules that target ryanodine receptors for the treatment of cardiac arrhythmia disorders. D.D. is consultant for OMEICOS Therapeutics, which develops drug molecules targeting the ω-fatty acid metabolism as an antiarrhythmic therapeutic strategy.

Author contributions

Both authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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